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EXPERIMENTAL INFECTION WITH *HÆMOPHILUS PERTUSSIS* IN THE MOUSE BY INTRANASAL INOCULATION.

F. M. BURNET AND CECILY TIMMINS.

From the Walter and Eliza Hall Institute, Melbourne.

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THE following observations have arisen as a result of a casual experiment to see what effect an emulsion of *Hæmophilus pertussis* would have when administered to mice by the method used for studies on the virus of influenza. An emulsion containing about 200 million organisms per c.c. was prepared in broth from a 48 hours' growth on Bordet-Gengou medium. Two drops (about 0·05 c.c.) were administered intranasally to two anæsthetized mice. Both mice appeared to be somewhat " off colour ", but showed no obvious symptoms when killed 5 days after inoculation. Post-mortem examination showed extensive œdematous consolidation of the greater portion of the lungs. The consolidated areas were of an almost uniform greyish-pink appearance, quite different from the plum-coloured consolidations of influenza virus infections. Exudate from the cut surface of one of the consolidated areas gave a profuse pure culture of *Hæmophilus pertussis* when plated on Bordet-Gengou agar (B.G.A.).

An examination of the available literature showed no reference to this phenomenon. Intratracheal inoculations of larger animals have been made fairly often without very noteworthy results. True symptomatic whooping-cough has apparently been produced only by inoculation of pure cultures of the Bordet-Gengou bacillus in children (Macdonald and Macdonald, 1933) and anthropoid apes (Rich *et al.*, 1936). Sprunt, Martin and Williams (1935) have recently described an interstitial broncho-pneumonia produced in rabbits by intratracheal inoculation of very large doses of *Hæmophilus pertussis*. They could not recover the organism from the lungs at periods beyond 48 hours after inoculation, so that the lesions may probably be regarded as resulting from an intoxication rather than from a true infection. Until recently, intranasal inoculation of mice under anæsthesia was not a recognized bacteriological

technique, so that it is not surprising that the method has not been applied to work with this organism.

From many points of view it would be desirable to have a convenient experimental animal in which a characteristic infection (not necessarily with " whooping-cough " as a symptom) could be induced by *H. pertussis*. From the practical point of view, such a means is particularly needed as a method of controlling the condition of cultures used for the preparation of vaccines, and as a test of their immunizing efficiency. Our experiments can be regarded only as preliminary, but they suggest that the infections produced in mice by intranasal inoculation with the organism represent the nearest approach yet recorded to a true specific infection in a convenient laboratory animal.

METHODS.

The standard Bordet-Gengou potato glycerine blood-agar (B.G.A.) was used for all cultures. With the exception of the first strain used, which was a culture isolated a few months previously and obtained from the Commonwealth Serum Laboratories, all the strains used have been isolated by ourselves from cough plates obtained from patients in the Children's Hospital, Melbourne, by the courtesy of the Medical Superintendent (Dr. S. W. Williams). Cultures were isolated by the usual methods, and their identity as *H. pertussis* established on a basis of the appearance of the colonies, morphology of Gram-stained organisms and agglutination by an immune serum prepared by immunization of rabbits with the first strain used. This serum has agglutinated all recently isolated strains to titre, and has no action on *H. influenzæ* strains. Its agglutinins, therefore, almost certainly correspond to the Phase 1 of Leslie and Gardner (1931).

Mice inoculations were made with suspensions from 3-day B.G.A. cultures. To give uniform results, it was found essential that the suspensions in broth should be prepared immediately before the inoculations were made. The content of bacteria in the suspensions was estimated by turbidity comparisons, a set of Burroughs Wellcome opacity tubes being used as standards.

The technique of inoculation will be described in detail, as the method is not widely familiar to bacteriologists. Young mice of about 20 g. weight are used. The animal is anæsthetized in a glass jar containing a pad soaked in a mixture of chloroform 1 part, ether 2 parts. It is important that the mouse should be breathing regularly without gasping during the inoculation, and we have found it advisable to place a loose layer of cotton-wool above the anæsthetic-soaked pad to prevent the mouse breathing too high a concentration of anæsthetic. When it is completely anæsthetized and breathing rapidly and regularly it is removed from the jar, grasped behind the neck with the thumb and forefinger of the left hand, and introduced into a glass-walled chamber of the type used in tissue culture. The inoculum is contained in a capillary pipette and administered to the mouse by depositing three small drops (about 0·05 c.c. in all) on the external nares, the head being held with the nose pointing almost vertically. If the mouse is properly anæsthetized, the inoculum is rapidly drawn in without bubbling, and only mice taking the

inoculation in this fashion should be included in the experiment. As it is impossible to avoid an occasional unsatisfactory animal which blows droplets of inoculum into the air, it is advisable to carry out the manipulations under a glass screen of some sort.

The mice were kept in separate glass jars and observed for 14 days in most experiments. In the earlier experiments, in which attention was directed mainly toward the extent and nature of the lung lesions, surviving mice were usually killed, and their lungs examined and cultured on the 7th day after inoculation.

RESULTS OF INTRANASAL INOCULATION OF *H. PERTUSSIS* IN MICE.

When large doses of bacteria are administered, death of the mice occurs in 2 or 3 days; with smaller doses death is proportionately delayed, but rarely beyond 11 or 12 days. Table I shows the results with graded doses of five recently isolated strains. There are no significant differences between the strains, nor is any falling off in virulence evident when a strain is kept on B.G.A. for some months. We have not yet developed any degraded (Phase III or IV) strains, and cannot state whether virulence is lost with these strains.

Mice dying early show general congestion and consolidation of the lungs, which may be macroscopically indistinguishable from those of mice dying early from experimental influenza virus infection. Usually, however, their colour is lighter, and variation in the intensity of reaction in different lobules gives a mottled appearance to many of them.

When mice receiving moderate amounts of culture die or are killed from the 5th day onwards, the most usual finding is an œdematous consolidation of the lungs, often complete, but sometimes involving only portions of the lungs. Mice examined very shortly after death may show evidence of acute terminal œdema in the form of bubbles in the trachea, and it is usual to find a slightly foamy fluid exuding freely when the consolidated regions are cut. As is to be expected, there are considerable variations in detail of the macroscopic appearance of the lungs in accordance with differences of dose administered, and with the presence or absence of secondary invaders.

Cultures from the cut surface of lungs showing consolidation have always been positive. *H. pertussis* may be present in pure culture or in association with other organisms, or, not infrequently, there may be a heavy growth of bacteria obscuring any *H. pertussis* colonies which may be present. It is interesting that a much higher percentage of pertussis lungs show secondary infecting organisms than do lungs infected with influenza virus lesions removed at about the same time after inoculation. No study has yet been made of the nature of the secondary invaders.

Table I shows that even with small doses, taking 10 or more days to kill, the lungs give a positive culture of *H. pertussis*. The lesions produced are therefore the result of a true infection, not a simple toxic effect of the bacterial products. It is in this respect that the present method differs from current "virulence" or toxicity tests of *H. pertussis* by subcutaneous or intraperitoneal inoculation in laboratory rodents.

TABLE I.—*Pathogenicity of Recently Isolated Strains for Mice when Inoculated Intranasally.*

Strain.	Dose in millions of 3-day culture.					
	1000.	200.	100.	40.	10.	8.	1·6.
Wi.	*2 2 3* 3	*2 2 2 2 2 3*	..	*2 3 3 4* S5 S11	..	4 *11* S5 S5 S11 S11	..
Ab.	*2* 3 3	..	*2 4 8*	..	*5 8* S11	..	..
Ba.	*2 13*	*2 2 2 2 3 6*	..	*2 3 3* 8 *14* S14	..	*4* 6 *7 9 10* S14	6 *11 11* 13 14 S14
Sk.	*4 4*	..	*2 4*	..	*4 6*	..	..
Bo.	2 *3*	..	7 *8*	..	*9* 14	..	..

In all the tables numbers indicate days after inoculation on which death occurred; S survived till, and was killed on the days shown. Lung cultures positive for *H. pertussis* are indicated by italics.

HISTOLOGY.

Sections from typical lungs removed 5 to 7 days after inoculation show a general interstitial pneumonia with cellular infiltration and œdema of the alveolar walls. Both mononuclear and polymorphonuclear cells are involved, the latter predominating. Portions of the lung may be completely airless, but patent alveoli are present in most regions. Here and there in the completely consolidated areas little clumps of close-packed small bacilli may

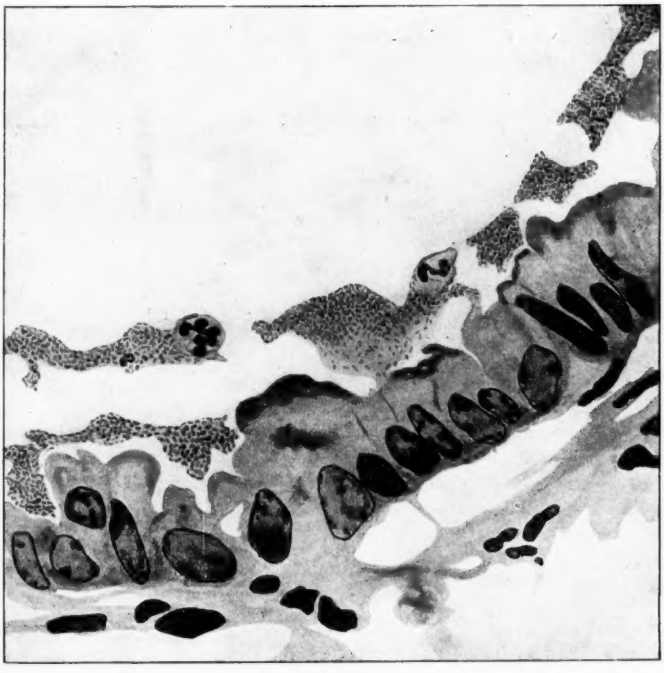

FIG. 1.—Section through wall of small bronchiole in a mouse killed 5 days after inoculation with *H. pertussis*, showing massive proliferation of small bacilli in mucus with destruction of the ciliated surface, but otherwise little cellular damage.

be seen, usually with an excessive polymorphonuclear infiltration in the vicinity.

The most characteristic lesions are in the bronchioles. The bronchiolar epithelium shows evidence of proliferation, and, judged by the numbers of goblet-cells, is actively secreting mucus. The regular arrangement of the cilia-bearing surface is completely lost. The cells project unequal distances into the lumen, and there are wedge-shaped gaps between cells. Close-packed masses of small bacilliform bodies, presumably *H. pertussis* proliferating in a film of mucus, lie over these cells or penetrate into the gaps between them.

The shape of some of these groups of bacilli suggests that they are probably growing in necrotic portions of epithelial cells as well as in the mucus.

Usually every section of a bronchus or bronchiole will show areas filled with bacilli, but some are more heavily involved than others. Smaller bronchioles may show an almost complete lining of this sort (Fig. 1). The tracheal epithelium is much less affected.

IMMUNIZATION EXPERIMENTS.

It is possible to produce a significant immunity to intranasal inoculation by immunization of mice either with living culture given intraperitoneally or with formalin-killed vaccines. The immunity is by no means absolute, and it is necessary to use two or more levels of test dose to obtain a sharp difference between immunized and control animals. Two protocols, which are representative of several other similar experiments, will be given.

In the first experiment two injections of approximately 100 million living organisms were given intraperitoneally at a week's interval, and immunity tested a fortnight later. Seven days after the test inoculations all surviving mice were killed, and their lungs examined and cultured. Table II gives the results. The most interesting feature of this experiment is the failure of the immunized mice to give positive cultures after inoculation with moderate doses, even when definite lesions were present. This finding was confirmed in two other experiments.

TABLE II.—*Immunization with Living Culture Given Intraperitoneally.*

Test dose.	Immunized mice.	Control mice.
10 million per c.c.	+, −, −	*7*, ±, ± ±
100 ,, ,,	++, −, −, −	*6*, *6*, ±±
1000 ,, ,,	*2*, ±±	..

Note.—In this experiment survivors were killed on the 7th day after inoculation, and the intensity of the lung lesions is indicated ++, + or ± ; − no consolidation.

Another series of mice was immunized with formalin-killed culture. A suspension in saline from three-day B.G.A. cultures of opacity equivalent to 1000 million organisms per c.c. was treated with 0·05 p.c. formaldehyde for 24 hours at 37°. The vaccine was then stored in the refrigerator, and mice inoculated subcutaneously or intraperitoneally with 0·5 c.c. for three injections at weekly intervals. Ten days after the last dose they were tested with controls by intranasal inoculation with the doses shown of living culture. The mice in this experiment were observed for 15 days and the survivors then discarded. The times of death and results of post-mortem lung cultures are shown in Table III.

With the larger dose the immunized mice showed only two survivors, but a greater average survival time than the controls. With the smaller dose there was a sharp distinction, all the immunized animals surviving, all the controls dying.

TABLE III.—*Immunization with Formalin-killed Vaccine.*

Method of immunization.	Test dose intranasally.	
	100 million per c.c.	10 million per c.c.
Form. vaccine 1 p.	*3 5* X S	X S S S S
S.C.	*3 4 4 6* S	X S S S S S
Control normal	*2 3 3 4*	*4 6 9 13*

Note.—Survivors in this experiment were observed for 15 days and then discarded without culture. X, death immediately after test inoculation.

DISCUSSION.

The Bordet-Gengou bacillus is almost universally accepted as the sole cause of whooping-cough, though some bacteriologists (Rich, 1932) may still maintain the possibility that an associated virus is involved to account for the high infectivity of the disease. An adequate explanation of the predominant symptom is provided by Mallory and Horner's (1913) discovery, frequently confirmed, of the presence of numerous bacilli entangled with mucus in the cilia of the bronchial epithelium. Paralysis of ciliary action by the toxic action of the bacteria prevents the normal transport of mucus and gives rise to the paroxysmal cough. Broncho-pneumonia is the chief fatal complication, and while other organisms usually predominate in post-mortem cultures, it is reasonable to consider that the lesion is initiated by the Bordet-Gengou bacillus.

Tests for pathogenicity of *H. pertussis* strains have been, up to the present, almost entirely confined to determining the toxicity of very large amounts of living or dead bacteria when administered intradermally or intraperitoneally in guinea-pigs or rabbits. In none of these tests is a true infection produced. The mouse lung lesion induced by intranasal inoculation of cultures shows the two cardinal features of the human pathology—multiplication of the organism in the mucus lining the bronchial tubes, with degeneration of the cilia-bearing portion of the epithelial cells, and interstitial pneumonia. The infection is frequently progressive and fatal, and the organism can almost always be re-isolated from the consolidated lung, often in pure culture. Within the limitations governing any similar work with a species for which the organism is not a natural pathogen, the mouse results offer a replica of the essential human pathology. One can regard the relation between the mouse and human infections as very similar to the relation between experimental pneumonia in the mouse due to influenza virus and human influenza.

Our results with immunization procedures need confirmation on a larger scale, but they are significant enough to suggest that the experimental method we have used may be of value for the standardization of *H. pertussis* vaccines intended for human prophylaxis.

SUMMARY.

1. Intranasal administration of *H. pertussis* cultures to anæsthetized mice results in characteristic pneumonic lesions, and in large doses is rapidly fatal.

2. Histological sections show an interstitial pneumonia and a typical

intense proliferation of bacilli in the mucus lying on the ciliated surface of the bronchial epithelium.

3. A significant degree of immunity can be demonstrated after intraperitoneal inoculation of living cultures or formolized vaccines.

4. The method may be of value in determining the antigenic efficiency of vaccines prepared for the prophylaxis of whooping-cough in children.

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AN INVESTIGATION OF THE INVASIVENESS OF A STRAIN OF *STREPTOCOCCUS HÆMOLYTICUS.*

M. G. PRADHAN.

From the Bacteriological Department, London Hospital and Medical College.

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THE purpose of this paper is to record the results of experiments made with the object of determining the factors responsible for the invasiveness of a strain of hæmolytic streptococcus. This streptococcus was isolated from a spontaneous outbreak of septicæmia in a stock of normal mice (Loewenthal, 1932*a*). It belongs to Lancefield's Group C, and as Loewenthal has shown (1932*b*, 1934), it can be dissociated into three variants. When first isolated the strain produced very small conical colonies on solid media and a granular growth in broth ; this form Loewenthal called N, signifying normal. From it two colonial variants were derived, the M form, giving a mucoid growth on solid media, and the O form, growing with a moist but non-mucoid colony. Both the M and O forms grow with a uniform turbidity in fluid media. A detailed study of these variants has been made, the results of which it is proposed to publish elsewhere ; for the purpose of this paper it will suffice to say that all three possessed the same virulence for mice.

In the past virulence and invasiveness have often been accepted as synonymous, but to-day the general opinion is that they are separate properties. However, in the case of a virulent bacterium which produces no soluble toxin these two properties must be closely related, and since this strain of streptococcus has not been found to elaborate an exotoxin, a study of the mean

whereby it invades the tissues of its host is of considerable importance and bears directly on the question of its virulence. Dennis and Berberian (1934) have demonstrated that the invasive properties of streptococci might be due to two types of action. Some strains inhibit the coagulation of the inflammatory exudate and so prevent the formation of a barrier to oppose their spread. Others not possessing this property are able to dissolve the coagulum, the capacity for lysis of the clot *in vivo* being correlated with the production of fibrinolysin *in vitro*. The strain of streptococcus under investigation appears, however, to possess no fibrinolytic activity. From the work of Van Deventer and Reich (1934) one would not expect a lysin to be produced for human fibrin, but it was not possible to elicit an action on mouse fibrin either, so that its invasiveness would not appear to depend on fibrinolysis. Duran-Reynals (1933) has demonstrated another factor, which would appear to be responsible for bacterial invasiveness. He has found that extracts of certain tissues, of testicle in particular, when inoculated intradermally along with particulate matter increase the area of spread of the particles. The spread of bacteria and viruses in the skin is facilitated by this action, which Duran-Reynals has found to be exercised also by extracts of staphylococci and streptococci. Goodner (1933) has shown the effect of pneumococcal lysates on the dermal infection of rabbits in experiments of a similar type, and since Duran-Reynals concluded from his work that " the power to invade the host's tissues depends upon the presence in the organism of a factor capable of increasing tissue permeability ", it was decided to investigate this aspect of the invasive power of the strain of the streptococcus in question.

EXPERIMENTAL.

Presence of Spreading Factor in 16–18-*hour Culture.*

The skin of a rabbit was used in testing for the spreading factor, since it was found too difficult to judge the spread in the skin of mice. 16- to 18-hours' cultures both on serum and serum-free media and their filtrates were tested according to the technique described by Duran-Reynals. At first indian ink was used as an indicator, but later 1 p.c. trypan blue (vital) was found to be more useful. Control inoculations were made with a similar quantity of dye diluted with distilled water instead of the extract. No spreading was seen with the 16-hour filtrates or the controls; only slight spreading could be observed with the 16-hour cultures (see Fig. 1).

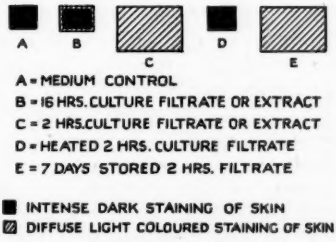
A
B
C
D
E
A = MEDIUM CONTROL
B = 16 HRS. CULTURE FILTRATE OR EXTRACT
C = 2 HRS. CULTURE FILTRATE OR EXTRACT
D = HEATED 2 HRS. CULTURE FILTRATE
E = 7 DAYS STORED 2 HRS. FILTRATE
INTENSE DARK STAINING OF SKIN
DIFFUSE LIGHT COLOURED STAINING OF SKIN

FIG. 1.—Diagrammatic representation of the spreading test.

Because of the relationship of invasiveness to infection it seemed reasonable to suppose that the bacteria would need the spreading factor to establish infection principally in the early stages of development. Several workers have observed or suggested that young cultures

are more invasive. Felty and Bloomfield (1924) found that the M.L.D. of a culture was smaller when the organisms were in the phase of active growth. Hare (1929) found that young cultures were more resistant to the bactericidal power of normal blood. He discusses the possibility of the cocci being capsulated, but could not demonstrate a capsule. Day (1933) suggested the formation of what he called a " V " antigen by virulent streptococci during the stage of active growth. He thought it to be some sort of secretion not only attached to the cocci, but also set free in the medium. Later Seastone (1934) demonstrated capsules in young cultures of virulent streptococci, and Ward and Lyons (1935) have confirmed this.

Formation of Capsules in Young Cultures.

Young cultures of the strain under investigation were examined for the presence of capsules when growing on meat infusion broth containing 20 p.c. rabbit serum; this concentration was found more suitable than 10 p.c. A large inoculum equal to one-tenth of the medium was made from an ordinary 18-hour culture. Cultures were examined in two ways: (1) A loopful was mixed on a slide with a similar quantity of a 10 p.c. colloidal silver solution and examined under a cover-slip. In such preparations capsules appeared as definite clear areas around the cocci. (2) Dried and fixed preparations were made and stained by Muir's method, which showed definite capsules, stained blue (Fig. 2). In such preparations some of the capsules were distorted or broken, suggesting that they were very fragile. Capsules were destroyed, and could not be seen if the young cultures were vigorously shaken on a shaking machine for half an hour. Peritoneal exudate collected from mice at hourly intervals after inoculation was examined by the same methods as were applied to the cultures. The time of appearance and disappearance of the capsules both *in vitro* and *in vivo* is recorded in the following table:

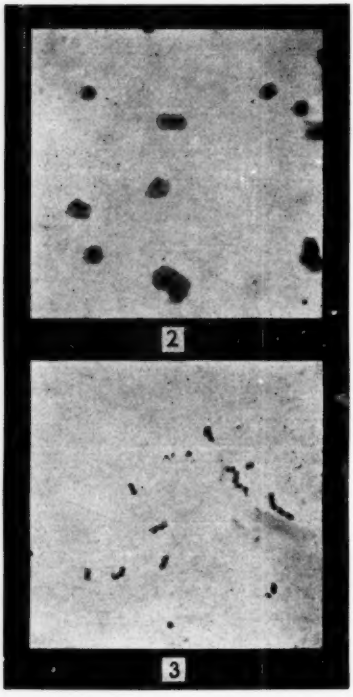

FIG. 2.—Capsulated cocci in young culture. Stained by Muir's method. × 1000.

FIG. 3.—Non-capsulated cocci in 18 hours old culture as control. Stained by Muir's method. × 1000.

	Capsules in serum broth. Variants.			Capsules in peritoneal exudate. Variants.		
	N.	O.	M.	N.	O.	M.
1 hour	—	—	—	—	—	—
2 hours	+	+	+	—	+	+
3 „	+ shrinking	+	+	+	+	+
4 „	±	+ shrinking	+	+	+	+
5 „	—	±	+	±	—	+
6 „	—	—	+ shrinking	—	—	+
7 „	—	—	—	—	—	+
8 „	—	—	—	—	—	—

It is clear from these results that this strain of streptococcus develops capsules in the early stages of multiplication both in artificial culture and in the animal body. They are well developed as a rule in 2 hours and persist up to the 7th hour at the longest. All three variants of this strain showed the same phenomenon, the only difference between them being the greater size of the capsule and its longer persistence in the M variant. No capsules were formed in tryptic digest broth cultures in the absence of serum. The development of capsules by this strain in the early stage of multiplication *in vivo* suggested that this was a factor concerned with invasiveness, and it was obviously of interest to find out if from the early capsulated forms a spreading factor could be obtained, and if so what relationship it bore to capsular material. The following experiments were made :

The Spreading Factor and Its Relation to Capsular Material.

Filtrate of 2-hour culture.—The N variant was grown in 20 p.c. serum broth for 2 hours, when microscopic examination showed that the cocci possessed well-developed capsules. The culture was then centrifuged and the supernatant fluid filtered through a Massen candle. The first half of the filtrate was rejected and the second half used in the test. A similar filtrate was obtained from another N form culture incubated for 16 hours, when no capsules could be detected. 20 p.c. sterile serum broth medium served as the control. To 0·45 c.c of each of these three fluids (the two filtrates and the medium) 0·05 c.c. of a 10 p.c. solution of trypan blue in distilled water was added. The concentration of the dye in the fluids was thus 1 p.c.

The fur on the flanks of healthy white rabbits was very closely clipped with an electric clipper without injury to the skin. This is important, as even the slightest injury to the skin causes a traumatic inflammation which interferes with the spread of the dye. With a No. 20 Record needle, 0·25 c.c. of each of these three fluids was injected intradermally at separate points in the skin of the rabbit's flank. A successful injection gave rise to a circular dark blue button-like wheal. Care was taken while withdrawing the needle from the skin not to allow any of the injected fluid to escape. The result was observed

after 16 to 18 hours, when it was seen that the 2-hours' filtrate had definitely aided diffusion of the dye. Inoculated with the medium alone the dye had not spread very far, and had produced a dark blue spot about 2 × 1·5 cm. with a more or less definite outline. The spot resulting from the injection of trypan blue and 16-hour filtrate was about the same size, though less sharp in outline, but where the dye had been introduced along with the 2-hour filtrate it had diffused throughout an area approximately 5 × 3 cm., which was less deeply stained. A diagrammatic representation of this is given in Fig. 1.

Spreading factor in saline extract.—Since shaking young cocci in saline deprived them of their capsules, it was decided to see if such an extract, which contained the capsular material, possessed also the spreading factor. The deposit obtained from 100 c.c. of 2-hour culture was shaken vigorously in 10 c.c. of normal saline on a shaking machine for half an hour. After this the mixture was filtered through a Seitz filter. A similar saline extract was prepared from an 18-hour culture. To these two filtrates 10 p.c. trypan blue was added to give a final concentration of the dye of 1 p.c. The control in this case consisted of saline and trypan blue.

Intradermal injections similar to those described above were made on the other flank of the same rabbits. All the results were noted at the same time. They were practically identical with those of the previous experiment in which culture filtrates were used (see Fig. 1). One rabbit was tested with filtrates obtained from all the three variants of this strain and in all cases with the same result.

Stability of the Spreading Factor.

One portion of a filtrate of 2-hour culture was heated at 56° C. for 1 hour and the other left at room temperature to serve as control. The heated filtrate and the control were tested for spreading factor. Heating destroyed the spreading factor (see Fig. 1). A filtrate that had been stored in the cold for seven days was tested and showed no loss of spreading capacity. Experiments with filtrates of young cultures on serum-free media which failed to show capsules also failed to show any spreading factor.

Influence of the Spreading Factor on Infectivity.

4 c.c. of 18-hour culture of Dochez N.Y. 5 in 20 p.c. serum broth were centrifuged; the clear supernatant fluid was set aside and the deposit was suspended in 10 c.c. of saline. This suspension was then distributed equally between four centrifuge tubes labelled A, B, C and D. These tubes were centrifuged and the clear supernatant saline removed. To the deposits in the four tubes 0·5 c.c. of the following fluids was added:

Tube A: 20 p.c. serum broth medium.

Tube B: Supernatant obtained from the Dochez culture.

Tube C: Massen filtrate from an 18-hour culture of the "O" form on 20 p.c. serum broth.

Tube D: Massen filtrate from a 2-hour culture of the "O" form on 20 p.c. serum broth possessing the spreading factor.

The bacterial deposits were well mixed with these suspending fluids and the four suspensions were inoculated intradermally into the shaved skin of the abdomen of a rabbit. It was noted 24 hours later that whereas suspensions A, B, C caused but small pin-point nodular lesions with a certain amount of redness around, suspension D, which alone contained the spreading factor, had produced a definite nodular lesion about the size of a small cherry, the skin around being diffusely red and swollen. The lesion began to regress after 24 hours.

Inagglutinability of Young Cultures.

These young cultures from which the spreading factor could be obtained were not agglutinated by immune sera possessing a high titre for the ordinary 18-hour cultures.

SUMMARY AND CONCLUSIONS.

It has been clearly demonstrated by these experiments that a spreading factor is elaborated by the cocci in the presence of serum during the early period of growth. That this substance is distinct from hæmolysin was shown by demonstrating that the 16-hour filtrate, having perhaps a slightly better hæmolytic potency than the 2-hour filtrate, did not possess any spreading factor.

As the growth proceeds this factor is either destroyed or neutralized, possibly by the products of metabolism of the cocci. It is only elaborated in media containing a high proportion of serum. The necessity of serum suggests that it can be produced even in greater proportions in the animal body where encapsulation of the cocci in the early stages of multiplication has also been found to occur.

As mentioned before, Day (1933) has suggested that his "V" antigen is intimately attached to the surface of the organisms and is also free in the medium. It would seem to follow from the present experiments that Day's "V" antigen is probably identical with the capsular substance of the young cocci which, when set free in the surrounding medium, constitutes "the spreading factor".

Young cultures were found to be inagglutinable by an immune serum of high agglutinin titre prepared against the usual 18-hour culture, and it seems reasonable to suppose that the young capsulated cocci are similarly resistant to the defence mechanism of the host.

The role of spreading factor in relation to infectivity is further brought out in the experiments showing the enhancing action which it exerts upon the power of an avirulent strain to produce an inflammatory lesion in rabbit's skin.

The factor responsible for the invasive power of this streptococcus would therefore seem to be the capsular material evolved by the cocci only in the very early stages of multiplication. Surrounding the cocci as a formed capsule this substance may enhance the resistance of the micro-organism to phagocytosis; free in the surrounding medium, it favours their spread through the host's tissues.

The author wishes to thank Dr. Loewenthal for his helpful advice during the course of this work.

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THE METABOLISM OF THE FILTER-PASSING ORGANISM "C" FROM SEWAGE.

ANTOINETTE PIRIE,

Associate's Research Fellow, Newnham College, Cambridge.

From the Biochemical Laboratory, Cambridge.

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IN 1936 Laidlaw and Elford described a group of small organisms which they had obtained from mixed London sewage. These organisms were just completely retained by collodion membranes of average pore diameter 0·25μ, which is also the limiting porosity for vaccinia virus (Elford and Andrewes, 1932). Cultures of the organisms also contained particles up to 0·5μ in diameter, but growth could be initiated by the smallest elements present. Three strains, separable serologically, were described. The most favourable medium for growth was Hartley's broth at pH 7·8 + 4·0 p.c. of Fildes' peptic digest of blood. Two strains could grow on broth alone, but for the third, called "C", blood seemed to be an essential addition to this medium.

Laidlaw and Elford say, "The chief interest in this group of micro-organisms seems to lie round the small forms which are to be found in all cultures. The existence of these suggests that the organisms may form another connecting link between the larger bacteria and the pathogenic viruses". With this idea in mind a study has been made of the biochemical activities of the organism "C". No separation into large and small forms has been practicable on the large scale necessary for such a study, so that the results reported here may

indicate the metabolism of the large forms. Since these are reproduced from the small forms it seems probable that their enzymes will be closely related.

For routine culture the organism "C" was grown in boiling tubes in 2·0 p.c. Difco proteose peptone at pH 7·8 plus 3·0 p.c. Fildes' peptic digest of blood. Twenty-four-hour cultures were used. After 24 hours in this medium the pinkish tinge of the blood disappeared and a faint cloud indicated growth. The small organism "C" also grew anaërobically, and under such conditions did not need blood or any other addition to the basal peptone medium. The best growth was obtained in the routine medium or in peptone + 0·04 p.c. hæmin + 0·1 p.c. glucose, but continued growth for at least ten generations could be supported aërobically in peptone + 0·1 p.c. glucose without hæmin. Peptone alone was unable to support growth under aërobic conditions. There was a very slight acid shift in pH during growth, particularly in those media containing glucose.

The chemical changes in the medium during growth were first investigated. Under aërobic conditions, growing in peptone and blood, the organisms did not change the ammonia, amino-nitrogen or reducing sugar content of the medium. If glucose were added a large part of it was utilized during growth. In a peptone + glucose medium without blood the glucose utilization was about half that in a medium containing blood. This may have been due to slower growth.

TABLE I.—*Utilization of Glucose by Growing Cultures.*

Time in hours.	Glucose p.c.	Total used per culture of 30 ml.	Medium.
0 .	0·212 g.	19·2 mg.	Peptone, glucose + blood.
24 .	0·148 g.		
0 .	0·203 g.	16·2 mg.	" " "
48 .	0·149 g.		
0 .	0·197 g.	8·7 mg.	" " "
24 .	0·168 g.	17·2 mg.	
48 .	0·143 g.		
0 .	0·145 g.	7·8 mg.	Peptone + glucose.
48 .	0·119 g.		
0 .	0·207 g.	9·9 mg.	" "
48 .	0·174 g.		

0·1 ml. *M*/1 glucose sterilized by filtration was added to 30 ml. of the medium after autoclaving. A sample was taken and kept at 0° C. during the course of the experiment. Glucose was estimated by the method of Hagedorn and Jensen (1923), 0·1 c.c. culture being used for each estimation.

Anaërobic Growth.

Under anaërobic conditions of growth in peptone the organism did not utilize glucose nor change the amino-nitrogen content estimated by formol titration, but the ammonia content of the medium increased. The presence of blood made no difference to this ammonia production. The pH of the medium shifted slightly to the alkaline side.

TABLE II.—*Ammonia Production During Anaërobic Growth.*

Medium.	Increase in ammonia nitrogen, mg. per ml.	Culture generation.
Peptone plus blood	0·016	1
	0·026	2
	0·020	3
	0·018	4
Peptone	0·025	1
	0·019	2
	0·021	3
	0·018	4

Ammonia was estimated by the method of Parnas and Heller (1924). A sample was kept at 0° C. during the course of the experiment. Estimations were all carried out in duplicate on 1·0 ml. of the solutions.

Metabolism of Organisms Centrifuged Out of the Medium.

Centrifugation for half an hour at 12,000 r.p.m. brought down the greater part of the organisms. From 32·0 ml. of culture a small deposit with a dry weight of about 1·0 mg. was obtained. The dehydrogenases and respiratory mechanisms of these centrifuged organisms were investigated. The sediment was rubbed up in a small amount of saline with a glass rod and then suspended in a known volume of saline or other medium. Table III shows the relative strengths of the dehydrogenases. Apart from an unknown substrate in peptone only reducing sugars were rapidly oxidized.

TABLE III.—*Dehydrogenases of Centrifuged Organisms.*

1·0 ml. suspension of organism "C", 1·0 ml. *M*/10 phosphate buffer pH 7·6, 0·2 ml. *M*/2000 methylene-blue, 0·1 ml. of a molar solution of the neutralized substrate were used per test.

Relative rates of reduction of methylene-blue, glucose being taken as a standard.

Glucose	100	Mannitol	25	Lactate	5
Fructose	100	Gluconate	0	Formate	0
Galactose	100	Sucrose	40	Succinate	0
Maltose	60	" Glycero-phosphate	75	Glycollate	0
Xylose	60	Pyruvate	30	Malonate	0
Arabinose	50	2 p.c. peptone	90	Malate	0

Respiration of centrifuged organisms was estimated in small constant volume Warburg manometers with factors of 0·4–0·5. The total volume of solution used per cup was 1·0 ml. Oxygen uptake was measured directly in the presence of phosphate buffer pH 7·6, and with 4·0 p.c. potassium hydroxide in the side bulb to absorb carbon dioxide.

Oxidation of Substances in Peptone.

Since 2·0 p.c. peptone is the basal medium for growth it was thought that it would contain substances oxidizable by the organism "C", but after growth on a peptone blood medium a suspension of organisms showed only a very

small respiration—sometimes none at all—in the presence of 2·0 p.c. peptone. Addition of hæmin or of blood to the suspension made no difference.

Oxidation of Glucose.

Table I shows that glucose is utilized by the growing organism. The rate of respiration of suspensions of organism " C " in the presence of glucose was therefore investigated. After growth on a peptone blood medium a saline suspension of the organism oxidized glucose *in vitro* very slightly. If the centrifuged organisms were washed with saline and re-centrifuged, no respiration whatever was shown in the presence of glucose alone. But if a small amount of hæmolysed rabbit blood or of a solution of hæmin was added as a respiratory catalyst a considerable oxygen uptake took place. Graph 1 shows the magnitude of this respiration in a typical experiment.

Therefore hæmin enables the organism to oxidize glucose which in the absence of the catalyst it would be unable to utilize. The demonstration of this reaction is very interesting because in the absence of hæmin the small organism " C " has a very low respiration in whatever medium it is suspended. The addition of hæmin transforms it into an actively respiring organism having the same type of metabolism as bacteria or protozoa.

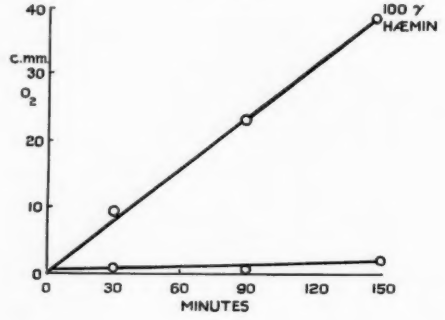
Lwoff (1934) found that certain protozoa which were unable to grow in a medium devoid of hæmin needed this compound to synthesize a respiratory system. It has long been known that some bacteria need hæmin for growth, and it is possible that in this case also it has a respiratory function (Knight, 1936). Lwoff found that different protozoa varied in their need for hæmin : *Leptomonas ctenocephali* needed 1 : 1000 blood, whereas *Strigomonas fasciculata* grew well in the presence of 1 : 300,000 blood. The organism " C " needs about 1 : 1000 blood for optimum growth, but will grow slowly on a peptone + glucose medium. Lwoff found that 5·0γ blood were enough to cause an increase of respiration and growth of *S. fasciculata* in the respirometer. The smallest amount of blood that will cause a definite increase in the oxygen consumption of the organism " C " was 50γ per 1·0 ml. of suspended organisms. With the concentration of organisms usually employed (about 0·1–0·2 mg. dry weight per 1·0 ml. suspension) the respiration was limited by the blood concentration if less than 1000γ blood per ml. organisms were present ; 50γ hæmin per ml. organisms was again the limit below which no definite effect on respiration was obtainable. The hæmin content of blood is 0·5 p.c., so that, per hæmin content, blood is roughly 200 times as active a catalyst as free hæmin.

Lwoff concluded that the protozoa formed the cytochrome respiratory system from added blood or hæmin. No evidence on this point was been obtained with the organism " C ". It rapidly destroys the hæmin, and after 24 hours' growth the colour of the medium is changed from pink to yellow-brown. This destruction is possibly correlated with the need for relatively large amounts of blood *in vivo* and *in vitro*.

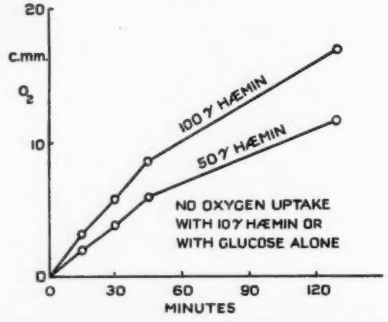
No sign of growth in the respirometer has ever been obtained. The respiration remains constant or diminishes over a period of hours. Laidlaw (1936) has estimated that the division time of the organism is about 1·0–1·6 hours,

so that if growth occurred an increase of respiration should be observed in an experiment lasting 3 hours. Such an increase was never obtained in any medium.

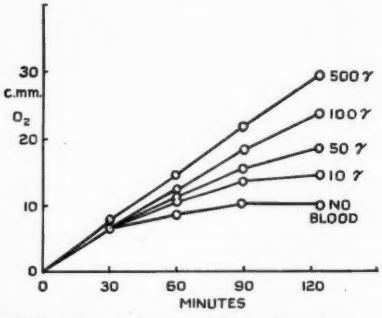
GRAPH 1.



GRAPH 2.



GRAPH 3.



GRAPH 1.—Oxygen uptake of organism "C". Catalytic effect of hæmin. 64·0 ml. culture centrifuged. 0·1 ml. *M*/10 potassium phosphate buffer pH 7·6, 0·05 ml. *M*/1 glucose, 0·7 ml. suspension of "C" added to each cup. 100γ hæmin added to one cup and an equivalent volume of saline to the other.

GRAPH 2.—Oxygen uptake of organism "C", with glucose and varying amounts of hæmin. 64·0 ml. culture centrifuged. Deposit washed with 16·0 ml. saline. 0·1 ml. *M*/10 potassium phosphate buffer pH 7·6, 0·1 ml. *M*/1 glucose, 0·7 ml. suspension of "C" and varying amounts of hæmin were used per cup.

GRAPH 3.—Oxygen uptake of organism "C" in the presence of glucose: catalytic effect of different amounts of blood. 64·0 ml. culture centrifuged. 0·1 ml. *M*/10 buffer pH 7·6, 0·1 ml. *M*/1 glucose, 0·7 ml. suspension of organism "C" in peptone added to each cup and varying amounts of hæmolysed rabbit blood.

The Q_{O_2} of organism "C" with glucose and hæmin was determined. A measured volume of culture was centrifuged in eight cups. The sediment from two cups was then used to determine respiration, and six cups were dried *in vacuo*, and the dry sediment was scraped out and weighed on a micro balance. The dried sediments were found to flake off the bottom of the centrifuge tube

satisfactorily. When respiring in the presence of $M/20$ glucose + 200γ hæmin per 1·0 ml. the Q_{O_2} was between 39 and 50. This value is rather higher than that for tissues, but well below the maximum values obtained for yeasts. An extended series of observations was not made, as it was only thought necessary roughly to establish the order of respiration.

$M/500$ cyanide inhibited 75 p.c. of the respiration with glucose in the presence of hæmin, and $M/1000$ cyanide caused a 50 p.c. inhibition. In these experiments filter-papers soaked in KOH plus KCN were placed in the side bulbs of the manometer vessels in order to avoid distillation of cyanide from the main solution (Krebs, 1935).

Glucose had been shown to disappear from the medium during growth, and in order to make certain that the oxygen uptake by suspensions of organism "C" in the presence of glucose and hæmin was really due to glucose oxidation, estimations of glucose in the suspension at the beginning and end of the experiment were made. Centrifuged organisms were suspended in peptone + glucose and hæmin; a sample was taken and kept in ice, and the respiration of 1·0 ml. measured in the usual way. At the end of the experiment another sample was taken and the change in glucose concentration during the course of the experiment was estimated. It was found that glucose disappeared, and that for every molecule of glucose oxidized three to four atoms of oxygen were absorbed. Glucose is therefore only partially oxidized by the organism. Further experiments in which gas pressure was compared in manometers with and without KOH in the side bulb showed that some carbon dioxide was produced during the oxidation of glucose. The rate of glucose breakdown in the manometer is much slower than in a growing culture. This is possibly due to the death of a large part of the organisms during centrifugation and during shaking in the manometer. If saline suspensions are left on the bench the enzymes frequently become quite inactive in half an hour.

Metabolism of Organism "C" Grown Without Blood.

The organism "C" can grow anaërobically in peptone alone or aërobically in peptone plus 0·1 p.c. glucose. The growth is never so great as it is in media containing blood. Organisms centrifuged out of such media show a slightly greater capacity to oxidize peptone than organisms grown on peptone + blood. They also take up extra oxygen in the presence of glucose if they are suspended in peptone. They do not do so if they are suspended in saline. The significance of the peptone is unknown. This extra oxygen taken up by the organisms in the presence of glucose + peptone—the medium in fact in which they have grown—is always small, amounting to between 3·0 ml. and 9·0 ml. per hour with the usual concentration of organisms. A large extra oxygen uptake, however, takes place if hæmin is added to the suspension. Therefore, these organisms, grown on a medium devoid of hæmin, are able to use it as a respiratory catalyst if it is added to the suspension of centrifuged organisms. Oxidation of glucose occurs rapidly in a saline medium + glucose + hæmin, the increase in oxygen uptake occurring about ten minutes after the addition of hæmin.

Flavoprotein prepared from yeast, lactoflavin and cytochrome C were all

inactive as respiratory catalysts. No extra oxygen uptake was observed in their presence with any substrate.

It must be assumed that the respiration shown by organism "C" in a manometer is a "resting" metabolism unconnected with growth, unless the dissolution of the organisms is proceeding at an equal rate. It has sometimes been considered that the failure of viruses to show any respiration, when separated from infected animals or from tissue cultures, is due to the fact that they do not respire unless they are actively dividing. The resting metabolism of the organism "C", which in some phases of its growth is as small as vaccinia virus, can easily be demonstrated if the appropriate respiratory catalyst is present. Without blood or hæmin there is no constant or significant oxygen uptake; in the same medium, but with the addition of blood or hæmin, the Q_{O_2} lies between 30 and 50. The organism seems unable to conserve the catalyst necessary for its respiration, and is dependent on the medium for a supply. It would be dangerous to draw any analogy between organism "C" and pathogenic viruses, since some of these have failed to show any respiration in the most diverse and complicated of media (Bronfennbrenner and Reichert, 1926; Schuler, 1935).

SUMMARY.

The metabolism of an organism isolated from sewage by Laidlaw and Elford (1936), which in some phases of its growth is as small as vaccinia virus, is described. The organism grows best in media containing blood. Organisms centrifuged from the medium oxidize glucose *in vitro* in the presence of blood or hæmin, but in the absence of one of these substances there is either no respiration or very little. No growth takes place during measurement of the respiration. Lactoflavin, flavoprotein and cytochrome C were unable to catalyse the respiration.

I have much pleasure in thanking Sir Patrick Laidlaw for sending me the strain of organism, and for much help, criticism and interest.

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THE METABOLISM OF THE FILTER-PASSING ORGANISM "A" FROM SEWAGE.

BARBARA E. HOLMES.

From the Biochemical Laboratory, Cambridge.

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LAIDLAW and Elford described three filter-passing organisms isolated from sewage which were serologically distinct and which differed in their habits of growth. The "A" and "B" strains were related serologically, though not identical, and "B" showed a greater tendency to grow in aggregated masses; "C" differed from them in having smaller colonies on solid media, and in requiring the addition of Fildes' digested blood to the ordinary nutrient medium (Laidlaw and Elford, 1936). It was thus of interest to determine whether the metabolism of those three organisms also showed differences. The metabolism of Laidlaw's "C" organism is described by Pirie (Pirie, 1937), while the results described in this paper were obtained with the "A" organism.

The first experiments were carried out with the dehydrogenase systems of the organism. The organism was spun out of 32 c.c. of culture on an Ecco Blitz centrifuge at 12,000 r.p.m., resuspended in tap-water saline and phosphate buffer and the appropriate substrate added. The dehydrogenase activity was then tested in Thunberg tubes with methylene-blue as an indicator. The substrates tried were the same as those described by Pirie for the "C" organism, which the "A" organism resembles in its power to reduce methylene-blue in the presence of meat broth, marmite, peptone solutions and even casein digests. It can also, like "C", reduce methylene-blue in the presence of glucose ($M/20$ final concentration), but differs sharply from "C" in that it can dehydrogenate lactic acid quite actively.

TABLE I.

"C" * organism.		"A" organism.
Glucose 100	.	Glucose 100.
Lactate 5	.	Lactate 100.
		Peptone 110 (one experiment).

Relative rates of reduction of methylene-blue (0·2 c.c. of $M/2000$ M.B.), taking glucose as the standard.

* Copied from Pirie's table.

This power of utilizing lactic acid may be useful to the organism when it is grown on Hartley's broth, which is likely to contain lactic acid, but since it will also grow very well on peptone (Difco proteose peptone), it can obviously obtain energy by other methods if necessary.

In order to give some idea of the magnitude of these reactions it may be said that 2 c.c. of an ordinarily active, unconcentrated culture grown on peptone and digested blood will reduce 0·2 c.c. of $M/2000$ methylene-blue in about 10 minutes.

The " A " strain, as Laidlaw points out, will grow on simple nutrient broths without the addition of digested blood or of glucose. In this way it differs from the " C " organism. I have found that it will grow on Difco proteose peptone without addition of any other substances, although when digested blood (Fildes, 1920) is added, the growth is improved. Similarly, the organism will grow anaërobically on peptone only, but growth is improved by the addition of blood. There is nothing in this to suggest that the digested blood is acting as a respiratory catalyst, and it seems likely that it improves growth chiefly because it contains nutritive substances which the organism can use. It can be shown that the organism quickly exhausts the hydrogen-donating substrates contained in the peptone or broth, and the amount of oxidizable substrate present is probably one of the factors limiting growth.

Growth was estimated as originally described for the bovine pleuro-pneumonia organism (Holmes and Pirie, 1932)—that is, by determining the length of time required for 2 c.c. of culture to reduce 0·2 c.c. of methylene-blue in a vacuum tube. A little extra peptone or broth was added in case the hydrogen-donating substrates should be used up during growth.

The following are typical results (" time " means simply the time required for the methylene-blue to become reduced):

2 c.c. culture grown on peptone and blood for 24 hours in air: time = 12 mins.

2 c.c. culture grown on peptone alone for 24 hours in air: time = 20 mins.

2 c.c. of the same culture tested without any extra peptone: time = 30 mins.

The last result shows that the substrates in the culture have been partly used up and that adding extra peptone therefore increases the rate of reduction; the following is another example of this kind:

2 c.c. culture grown on broth for 44 hours. Extra broth added: time = 18 mins.

2 c.c. culture grown on broth for 44 hours. Saline added: time = 27 mins.

The following figures refer to cultures grown anaërobically for 48 hours. It is interesting to see the almost complete exhaustion of oxidizable substrate in the cultures. In spite of this, estimation showed that no ammonia had been produced during growth:

2 c.c. culture grown on peptone only. Saline added: time = ∞.

2 c.c. culture grown on peptone only. Extra peptone added: time = 30 mins.

2 c.c. culture grown on peptone and blood. Saline added: time = 100 mins.

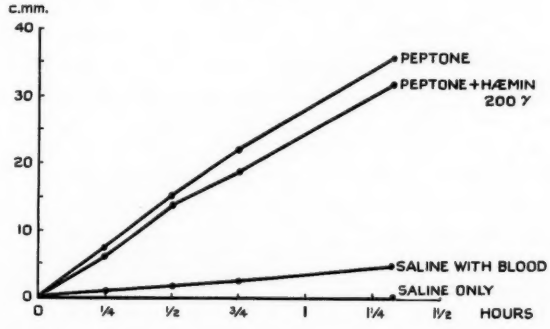
2 c.c. culture grown on peptone and blood. Extra peptone added: time = 23 mins.

What the substance in the peptone medium is that can be utilized by the

" A " organism is not known, but measurements of respiration show that there is a substrate in the peptone which the organism can oxidize. For the purpose of estimating oxygen uptake, cultures were centrifuged as previously described. 32 c.c. of culture grown in peptone without added blood yield enough deposit to give an easily measurable oxygen uptake in two small Warburg manometers. The addition of digested blood to the cultures was avoided in order that the centrifugate should not contain hæmatin.

Fig. 1 shows that, unlike the " C " organism, the " A " organism gives a good oxygen uptake with added peptone. This agrees with what is known of the growth habits of the two organisms, " A " being capable of reasonably good growth in air on peptone only, while " C " requires the addition of digested blood or glucose. In order to show the oxygen uptake satisfactorily, the final strength of peptone in the manometer cup should be about 4 p.c. This

FIG. 1.



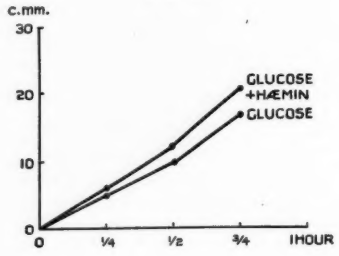
secures that sufficient substrate is present, and also protects the organism, which may be fragile in saline alone; the agalactia organism and the " C " organism have both been found to die quickly in saline, and many workers report that this is also true of the vaccinia virus.

It will be seen that the addition of a fair amount of blood (0·1 c.c. in 1 c.c.) gives a very small oxygen uptake; this can sometimes be seen in the presence of peptone, but usually not if enough peptone is present to give a good respiration. The addition of hæmin or dilute blood does not give any increased uptake. There is therefore certainly no question of any catalytic action of hæmin affecting the oxidation of peptone. This is reasonable in view of the fact that the organism can grow on peptone alone, and since it shows a small respiration in the presence of blood alone, and also a small increase in the rate of methylene-blue reduction (on one occasion, for instance, organism + saline took 61 minutes to reduce 0·2 c.c., organism + saline + blood took 43 minutes), these respiration experiments support the idea that the effect of blood in improving growth may be due chiefly to the nutritive materials it contains.

It is impossible at present to say how the oxidation of the peptone proceeds, or what substance contained in the peptone is being oxidized. Like " C ",

" A " does not produce any ammonia during growth in air, but unlike " C ", it does not produce any considerable amount of ammonia even when grown anaërobically in peptone. Attempts were made to decide whether any CO_2 was produced during growth on peptone and blood. The results were quite negative, but it is likely that the amount of CO_2 produced by respiration during growth would fall within the limits of experimental error, since the amount of CO_2 already present in the peptone is considerable. An experiment done in the Warburg apparatus with organisms concentrated by centrifuging shows that at least some CO_2 is produced. In this experiment the oxygen uptake was 32 c.mm. in $1\frac{1}{4}$ hours when potash was present in the side bulb, while the movement of the manometer corresponded to 18 c.mm. in the same time in the absence of potash. It should be explained that the manometer cups are small, having factors of about 0·5, so that amounts of this sort can be measured quite easily.

FIG. 2.



It cannot be assumed that the metabolism in the Warburg manometer where the medium is well aërated is the same as it is during growth in a rather deep tube of liquid medium. In fact, although the " A " organism appears to be able to oxidize glucose in the manometer (see Fig. 2), and can also dehydrogenate glucose in the presence of methylene-blue (Table I), it has not been possible to demonstrate the disappearance of glucose during growth. That is to say, that in cultures to which glucose has been added there is no fall in reducing value during growth.

In the conditions in which we have cultivated these organisms, the metabolism of " A " organism described here differs from that of the " C " organism described by Pirie (1937) in the following respects: It can dehydrogenate lactic acid, and it shows a good respiration in the presence of peptone only; on the other hand, it does not form ammonia when grown anaërobically, and does not cause the disappearance of glucose during growth in air.

These two organisms, like the " viruses " of pleuro-pneumonia and agalactia (Galloway, 1930; Holmes and Pirie, 1932; Pirie and Holmes, 1933; Pirie, 1934) have an easily detectable respiratory or fermentative metabolism, by which they are no doubt able to obtain their energy for growth. Actually these four organisms can be distinguished from each other by their metabolic processes in the same way as many bacteria can be distinguished. The smallest forms of these organisms are not very different in size from the largest

true viruses, like vaccinia. So far workers with the true viruses have not been able to demonstrate any energy-yielding metabolism (see Schuler, 1935). I have attempted to do this with vaccinia growing in tissue cultures, without being able to detect any change of metabolism due to the virus infection of the culture. In a recent experiment vaccinia virus scraped off the skins of two rabbits (according to the method of Parker and Rivers, 1935) failed to give any respiration with a mixture of peptone, glucose and blood. It may be that this apparent lack of respiratory metabolism is connected with the complete inability of the true viruses to live independently of tissues—even in the work of Eagles (1935) the virus was cultivated in a strong tissue extract—or it may simply be that large enough amounts of living organisms have not yet been used. It is shown by skin-tests in rabbits that a large proportion of the 1 mg. or so of vaccinia virus that can be obtained from one rabbit's skin must be inactive or dead; 1 mg. of living virus would give a titre much higher than that usually obtained.

SUMMARY.

The "A" filter-passing organism cultivated from sewage by Laidlaw and Elford has been studied. Its metabolism is shown to be different in several respects from that of the "C" organism from sewage.

It will grow on a simple peptone medium, and will respire in the presence of peptone. It does not form ammonia from peptone during growth; it soon exhausts the supply of hydrogen-denoting substances in the peptone when growing.

I have much pleasure in acknowledging that I am in receipt of a personal grant from the British Empire Cancer Campaign.

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THE SALT OPTIMUM IN ANTIBODY-ANTIGEN REACTIONS.

J. T. DUNCAN.

From the Division of Bacteriology and Immunology, London School of Hygiene and Tropical Medicine.

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THE ANTIBODY, ANTIGEN AND SALT OPTIMA.

For any effective combination of two of the three reagents concerned in agglutination, the immune serum, the bacteria and the salt, an optimal quantity of the third reagent, for the highest velocity of agglutination, can be determined by titrating it in a series of varying concentrations against constant amounts of the other two. The optimal quantities, determined in this way, will be referred to as the *antibody optimum*, the *antigen optimum* and the *salt optimum* respectively.

The antibody and antigen optima have been discussed already by Duncan (1934), who arrived at the following interpretations based on tests made with *Bact. dysenteriæ* Flexner Z and the homologous immune serum. The *antibody optimum*, which is determined by titrating diminishing concentrations of immune serum against constant amounts of bacteria and salt, as in the common form of the agglutination test, and reading the earliest point of agglutination, indicates the most *effective* antibody sensitization of the bacteria, although larger proportions of antibody combine with the organisms at higher serum/suspension ratios. It coincides with the zone of *maximum cohesion* of the agglutinated particles, as judged by the resistance of these particles to dispersion by mechanical force, and it represents an equilibrium between the combined and uncombined antibody; for a constant proportion of the total antibody, equal to approximately 7 p.c., remained free in the supernatant after agglutination of the dysentery bacillus. It should be noted that this optimum is analogous, in the method of its determination, to the Ramon (1922) optimal ratio of antitoxin to toxin and to the " constant-antigen optimum " of Dean, Taylor and their colleagues (Taylor, 1933).

The *antigen optimum*, found by titrating a series of rising concentrations of the bacterial suspension against fixed amounts of serum and salt, and noting the mixture which gives earliest agglutination, has a much lower serum/suspension ratio than the antibody optimum. It does not correspond with the zone of maximum cohesion of the particles, but it seems to depend upon the optimum density of suspension for the highest incidence of *effective* contacts between the sensitized bacteria. At densities of suspension greater than optimal, the serum amount being constant, the consequent fall in sensitization of the organisms outweighs the advantage of the increased rate of contacts by rendering a high proportion of these contacts ineffective, and at densities less than optimal, although the antibody sensitization of the organisms may

be better, the fall in the rate of contacts lowers the velocity of agglutination. This optimum corresponds to the " constant-antibody optimum " of Dean and Webb (1926), and, in connection with the value of the latter optimum in quantitative estimations, it may be significant that the antigen optimum, determined for various O antibody-antigen systems, was always in close proximity to that serum/suspension ratio which marks the limit (end point) of complete agglutination of the bacteria.

The *salt optimum*, the subject of the present work, may be determined by adding uniform quantities of the immune serum and bacterial suspension to a series of diminishing concentrations of salt, starting from a relatively strong concentration. With flagellated bacteria optimal agglutination is usually sharply defined. Measurement of the antibody residues in the supernatant liquids after agglutination shows the zone of *maximum antibody absorption* to be coincident with, or very close to, that of *optimal agglutination*.

It seems, therefore, that the antibody optimum gives that antibody-antigen compound which results in the greatest cohesion of the agglutinated particles ; that is to say, it affects the *quality* (firmness) of agglutination. The salt optimum gives the highest ratio of antibody/antigen in the compound, and affects the *quantity* of the reaction as measured by the highest agglutinating serum titre. The antigen optimum, depending upon the highest concentration of adequately sensitized bacteria, affects chiefly the stage of particulation ; that is to say, it determines the velocity of agglutination.

THE INFLUENCE OF THE SALT OPTIMUM ON THE ANTIBODY-ANTIGEN REACTION AND ON AGGLUTINATION.

Test Materials.

Dialysed, salt-free, sera and suspensions were used for determination of the salt optimum and all tests involving low salt concentrations. The precipitate of " euglobulin " which formed in the early stages of dialysis of the serum, and a second precipitate formed on heating the clear dialysed serum to 48°–55° C., were discarded. A variable proportion of the antibody, up to about 50 p.c., was lost with the euglobulin precipitate, but no appreciable loss resulted from the heat precipitation. The reaction of the dialysed serum, which varied with the extent of the dilution through endosmosis, was between pH 5·6 (bromo-cresol-purple) and pH 6·3 (bromo-thymol-blue). In the actual tests the reaction of the mixtures was between pH 6·8 and pH 7·0.

The only salt used was sodium chloride, which will be referred to throughout the paper as " salt ".

For convenience of description the term " antibody-antigen reaction " is restricted to the combination or dissociation of antibody and antigen, and is not used for the visible (second) stage of agglutination and precipitation.

The Salt Optimum in the H Form of Agglutination.

Table I illustrates the results of a test to determine the salt optimum for the H form of agglutination, with a *Bact. typhosum* immune serum and a *Bact. Stanley* suspension ; both dialysed. The test is designed to show the movement of the salt optimum with variations in the ratio suspension/serum.

TABLE I.—*Salt Optima Determined for Several Combinations of* Bact. typhosum *Immune Serum and* Bact. Stanley *Suspension (Both Dialysed).*

	Serum dilutions.	Suspension millions of organisms per c.c. of mixture.	Ratio millions of organisms to 1·0 c.c. of serum.	Salt concentration (molar): *M*/14.	*M*/28.	*M*/56.	*M*/112.	*M*/224.	*M*/448.	*M*/896.	0.
A	1/3	500	1500	+	+	+	+	+	+	+	+
B	1/12	500	6000	+	+	+	+	+	+	+	−
C	1/48	500	24,000	+	+	+	+	+	+	−	−
D	1/192	500	96,000	+	+	+	+	−	−	−	−
E	1/384	125	48,000	+	+	+	+	−	−	−	−
F	1/96	500	48,000	+	+	+	+	+	−	−	−
G	1/24	2000	48,000	+	+	+	+	+	+	+	−
H	1/24	500	12,000	+	+	+	+	+	+	+	−
I	1/24	125	3000	+	+	+	+	+	+	+	−

A + sign indicates agglutination; a thick unbroken rule indicates position of optimal agglutination; a thick broken rule indicates position of maximum antibody absorption.

From A to D the suspension dose is constant, but the serum dose varies, giving an increase in the ratio suspension/serum as the serum is diluted, and it will be seen that optimal agglutination moves to a higher salt concentration as this ratio increases. This movement is not a result merely of dilution of the serum, for in E, F and G the serum and suspension amounts vary together, so that the ratio suspension/serum remains unchanged, and the salt optimum is constant at 1/56 *M*. In G, H and I the ratio suspension/serum is lowered by decreasing the suspension amount, while the serum dose is unchanged, and the salt optimum moves to a lower concentration. It will be shown later that the rise in the salt optimum with the increase in the ratio suspension/serum does not continue beyond a salt concentration of about 1/56 *M* or 1/28 *M*. This point, which is not material to the main issue of the present work, will be dealt with in a subsequent paper.

So far as the position of maximum absorption of antibody has been determined, it agrees closely with that of optimal agglutination. It will be noticed that the lowest effective salt concentration for agglutination rises with the dilution of the serum (A to D and G to E), and this movement is independent of the suspension/serum ratio (G to E). Eventually, as shown in Fig. 3, the lowest effective salt concentration will coincide, at the serum end titre of agglutination, with the salt optimum, and at this critical point aggutination does not occur at any other salt concentration.

Fig. 1 illustrates the agglutination of a suspension of *Bact. typhosum* by the homologous immune serum (both dialysed) in various dilutions and at various salt concentrations, the dose of suspension being constant. The

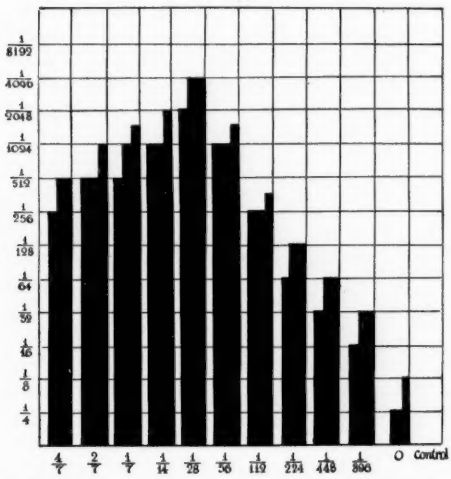

FIG. 1.—Agglutination (H form) of a constant mass of *Bact. typhosum* (500 million organisms in 1·0 c.c.) by various dilutions of the homologous immune serum (both dialysed salt-free) at various salt concentrations. The figures on the ordinates represent concentrations of the serum, and those on the abscissæ fractions of a molar concentration of the salt. The widest part of the shaded column (except the salt-free column) indicates *complete* agglutination and the narrower parts diminishing degrees of agglutination. In the control, *Bact. dysenteriæ* (salt-free) was substituted for *Bact. typhosum*.

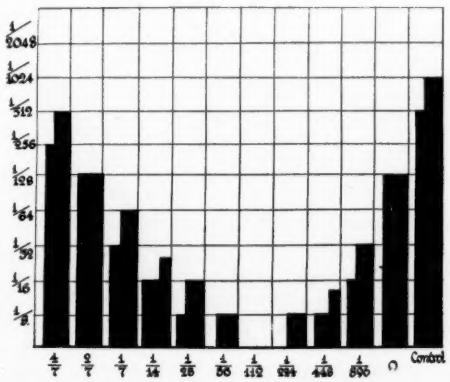

FIG. 2.—Measurement of the antibody residues in the supernatants of the 1/4 serum row of Fig. 1, by agglutination of a *Bact. typhosum* suspension in 1/7 *M* salt solution. The figures and shading have the same significance as in Fig. 1; those on the abscissæ indicate the salt concentrations of the original agglutination test.

highest serum titre of agglutination is reached at a salt concentration of 1/28 *M*. This result agrees in its more important features with that of a similar test made by Northrop and De Kruif (1922), but these observers attributed the end-point curve, which reached its acme at a salt concentration of 0·1 *N*, to the effects of the salt on the so-called second phase of agglutination. The results of the present work, however, indicate that the serum end-points of agglutination are determined by the amounts of antibody combining with the

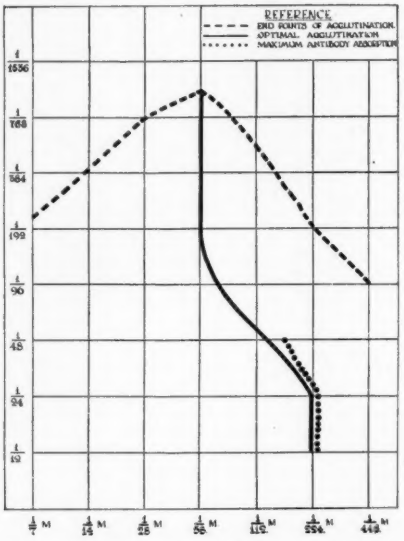

FIG. 3.—Agglutination of a constant mass of *Bact. Stanley* (500 million organisms in 1 c.c.) by various dilutions of *Bact. typhosum* immune serum (both dialysed salt-free) at various salt concentrations; to show the relation of the *salt optimum* to the serum end-titre agglutination curve. The figures have the same significance as in Fig. 1.

bacteria at the different salt concentration. It will be noticed that agglutination occurs even in the absence of salt at very high serum concentrations; this matter will receive attention in a subsequent paper.

After standing overnight in the cold, the supernatants from the 1/4 serum row were collected by centrifuging and tested for free antibody residues with a suspension of *Bact. typhosum* (500 million organisms in 1·0 c.c.) The measurements of these residues in terms of agglutination titres are given in Fig. 2. If the quantity of the supernatant antibody is accepted as an indication of the amount of antibody absorbed by the organisms, it will be seen that the agglutination end-point curve corresponds fairly closely with the curve of the antibody-antigen reaction, except that the two maxima, agglutination at 1/28 *M* and antibody-antigen combination at 1/112 *M* salt, do not coincide. It will be noticed, however, that the maximum agglutination was read at a serum dilution of 1/4096, while the maximum antibody-antigen reaction was determined at a dilution of 1/4, and it was been shown in Table I (A to D) that the salt optimum

at low serum dilutions moves to a higher salt concentration as the serum dilution rises, the suspension amount remaining constant.

Fig. 3 illustrates the movement of the salt optimum, determined by agglutination, from the low serum dilutions to the agglutination end-point peak. The movement to higher salt concentrations with the rise in the ratio suspension/serum ceased when the salt concentration 1/56 *M* was reached. The position of maximum antibody absorption cannot be followed into the higher serum dilutions, but so far as it can be traced it agrees fairly closely with that of optimal agglutination.

The test illustrated in Figs. 1 and 2 was made eighteen months before those in Table I and Fig. 3, and the suspensions used were different, but numerous tests with H antibody-antigen systems have shown that in the matters under consideration all such systems behave similarly; so the results shown in Table I and Fig. 3 are comparable with those in Figs. 1 and 2. The optimal salt concentration at the serum end-point agglutination peak seems to vary in tests with different immune combinations; Northrop and De Kruif (1922), using *Bact. typhosum* suspension and immune serum, found it to be 0·1 *N* salt, and in the present work it was 1/28 *M* with the *Bact. typhosum* suspension and serum (Fig. 1), and 1/56 *M* with the *Bact. Stanley* suspension and *Bact. typhosum* serum (Fig. 3); but so far as the present experience goes it seems to be constant at the figures stated for these two immune combinations.

Notwithstanding the accepted rôle of the kation in removing any obstacle to agglutination presented by a surface potential, the results of the foregoing tests support the view that the *principal* effect of the salt in agglutination is on the combination of antibody with antigen.

Other Systems Used.

Results similar to those illustrated in Figs. 1 and 2 were got with *Bact. œrtrycke*, *Bact. Stanley* and *Bact. Gärtner* suspensions and the corresponding immune sera (H antibodies).

Soluble antigens.—Three years ago, in the course of precipitin tests with extracts of fungi, two tests similar in arrangement to that shown in Fig. 1 were set up with two different yeast extract antigens and the homologous precipitating sera. The limits of precipitation followed a similar course, but at much lower serum titres, to that shown for H agglutination in Fig. 1, and the highest serum dilution giving precipitation was reached, in each case, at a salt concentration of 1/28 *M*. This experiment was not taken further.

THE EFFECTS OF SALT CONCENTRATIONS GREATER AND LESS THAN OPTIMAL ON DISSOCIATION OF ANTIBODY-ANTIGEN.

Heidelberger, Kendall and Teorell (1936) found that the amount of antibody nitrogen in precipitates formed from similar mixtures of Type III pneumococcus SSS. and the homologous antibody at different salt concentrations diminished as the salt concentration was increased from 0·1 *M* to 1·79 *M*. They consider this fall to be due to a shift in the reaction equilibrium by which a given amount of the antigen combines with less antibody in the presence of

higher salt concentrations than under ordinary conditions. This result is comparable to that shown in the left-hand side of Fig. 2. In a later paper Heidelberger and Kendall (1936) show that the shift in the reaction equilibrium is reversible, and by transferring to strong salt solution a washed precipitate formed at a lower salt concentration they were able to obtain a relatively pure solution of dissociated antibody.

In the earlier stages of the present work liberation of H antibody had been observed when strong salt solution was added to agglutinated typhoid bacilli, but this observation was not followed up at the time. In view, however, of the observations of Heidelberger and his associates on the reversibility of the salt effect on the antibody-antigen reaction, mentioned above, and the demonstration of an optimal salt concentration for the antibody-antigen reaction (Fig. 2), it was decided to determine the relation of the salt optimum to the dissociation of antibody-antigen.

The following tests were therefore made of dissociation of H antibody, at various salt concentrations in the hyper-optimal and hypo-optimal ranges, from a suspension of *Bact. Stanley* sensitized with typhoid H antibody.

Firstly, the salt optimum was determined for the serum-suspension mixture to be used. Then this mixture was set up at the salt optimum (1/112 *M*) and at various salt concentrations in range from sixty-four times (4/7 *M*) the optimum down to one-tenth the optimum. After agglutination the flocculi were redispersed and samples immediately taken from the different tubes. These samples were centrifuged and the free antibody content of the supernatants measured by the agglutination end-titre for a *Bact. Stanley* suspension. The results agreed closely with those in Fig. 2 ; showing a progressive increase in the amount of uncombined antibody on both sides as the salt concentration deviated from the optimum, which had little or no free antibody in its own supernatant.

The remainders of the dispersed initial mixtures were then distributed each into four tubes in equal volumes. To Tube I was added salt solution of much higher concentration than that of the initial mixture ; to Tube II several volumes of either very dilute salt solution or distilled water ; Tubes III and IV were used as controls of the effects of simple dilution, and received salt solution of the same concentration as that of the initial mixture in volumes equal to those of the liquids added to Tubes I and II respectively. The various sets of tubes were then returned to the water-bath and, some time after re-agglutination, the supernatants were collected by centrifuging and their antibody content measured.

The results of these tests showed :

A. No appreciable dissociation of antibody in the control mixtures diluted with salt solution of the same concentration as that used at the initial sensitization.

B. At the salt optimum and at all salt concentrations greater than this the addition of a *stronger* salt concentration than that of the initial sensitization caused dissociation of an amount of antibody, increasing with the difference between the initial and final salt concentrations. The addition of *weaker* salt concentrations than the initial led to a reduction in the amount of supernatant antibody, due to additional absorption of antibody, provided that the

final salt concentration was not less than the optimum; the amount of additional antibody absorbed increased with the difference between the initial and final salt concentrations.

c. At the salt optimum and at all concentrations less than this, dilution with very *weak* salt solution or distilled water caused dissociation of antibody, while the addition of a *stronger* salt than the initial (except at the optimum) led to further antibody absorption, until the final salt concentration equalled the optimum. In both cases the total effect was related to the difference between the initial and final salt concentrations.

Other tests were made with sensitized bacteria freed from supernatant serum by centrifuging and washing very gently in salt solution of the strength used at their sensitization. They were then dispersed in salt solutions of various strengths and allowed to re-agglutinate; afterwards the supernatants were collected and tested for their content of free antibody. These tests showed that dispersion of the organisms in salt solution of the same strength as that in the sensitizing mixture caused no appreciable dissociation of antibody. Organisms sensitized at the salt optimum yielded antibody when dispersed in stronger or weaker salt solutions. Organisms sensitized at a concentration above optimal yielded antibody only when dispersed in stronger salt solution, and those sensitized at concentrations below optimal, only when dispersed in a weaker salt solution or distilled water. The amount of antibody dissociated increased with the difference between the salt concentration used at sensitization and the final concentration.

All of these results may be summarized as follows: The antibody-antigen reaction reaches its maximum at the salt optimum and diminishes progressively as the salt concentration deviates in either direction away from the optimum. When motile bacteria are sensitized with antibody at a certain salt concentration, and, afterwards, that salt concentration is raised or lowered, antibody will be dissociated from the bacteria if the final salt concentration deviates further than the initial away from the optimum; and additional antibody will be absorbed if the change brings the final salt concentration nearer to the optimum, or possibly, if free antibody is not available, the stability of the antibody-antigen compound may be increased.

Relatively large amounts of dissociated H antibody may be obtained by dispersing washed organisms sensitized at or near the salt optimum in either very strong salt solution (4/7–16/7 *M*) or in distilled water.

THE SALT OPTIMUM IN THE O FORM OF AGGLUTINATION.

Duncan (1934) found that the effects of variations in the salt concentration on the O form of agglutination (*Bact. dysenteriæ*) differed from those on the H form of agglutination (*Bact. typhosum*) described by Northrop and De Kruif (1922). This observation has been confirmed since then with all the H agglutination systems referred to above and the O systems represented by *Bact. dysenteriæ* W, X and Z, *Bact. pullorum* and non-motile variants of *Bact. typhosum* and *Bact. aertrycke*, as well as with pure O agglutination of flagellated bacteria.

Fig. 4 illustrates the agglutination of *Bact. dysenteriæ* Z by various dilutions

of the homologous serum at various salt concentrations. The figure includes two separate tests; in one dialysed serum and suspension were used so that the effects of the weakest salt concentrations could be determined, and in the other undialysed serum was used in order that the effects of strong salt solutions at the highest concentrations of antibody available could be observed. The serum dilutions stated refer only to the undialysed serum; the dilutions of the dialysed serum are not given, but the table is so arranged that the antibody contents of the two sera, reading across, are approximately equal. With this provision the figure may be accepted as illustrating the result of a single test. The general outline of agglutination agrees with that of tests in which

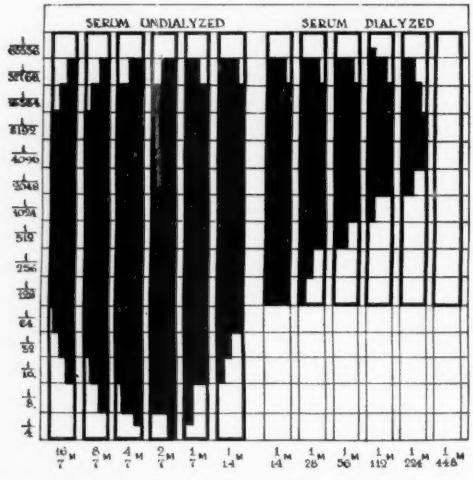

FIG. 4.—Agglutination of *Bact. dysenteriæ* Z (600 million organisms in 1 c.c.) by various dilutions of the homologous immune serum at various salt concentrations. The figures and shading have the same significance as in Fig. 1.

only dialysed serum was used, except that in these tests, owing to the reduced antibody content of the dialysed serum, the movement of the lower salt limits of agglutination with the rise in the serum concentration could not be followed beyond a concentration of 1/56 or 1/28 *M* salt.

If the serum end-titres of agglutination at the different salt concentrations are compared it will be seen that, in contrast with Fig. 1, there is little variation; there is weakening of end-titre agglutination at the strong salt end and a gradual improvement in the reaction towards the weak salt end, culminating in a slight rise in the end titre at 1/112 *M* salt followed by a fall at 1/224 *M*.

The most striking feature of this and all tests with O agglutination systems is the progressive rise in the lower salt limit of agglutination as the serum dose increases until a salt concentration of 2/7 *M* is reached; this concentration, which has been reached but not exceeded in several tests, seems to be significant. The failure of agglutination by strong serum at weak salt concentrations

is due to a diminution of the antibody-antigen reaction: at the *strongest* serum doses antibody absorption is greatest at the highest salt concentrations and falls progressively with dilution of the salt.

At salt concentrations greater than 2/7 *M* agglutination is weakened or absent in the strong serum range (dils. 1/4–1/32). This inhibition is not the result of lessening of the antibody-antigen reaction, which, as just stated, reaches its maximum in this region; but it seems to be due to an "anti-

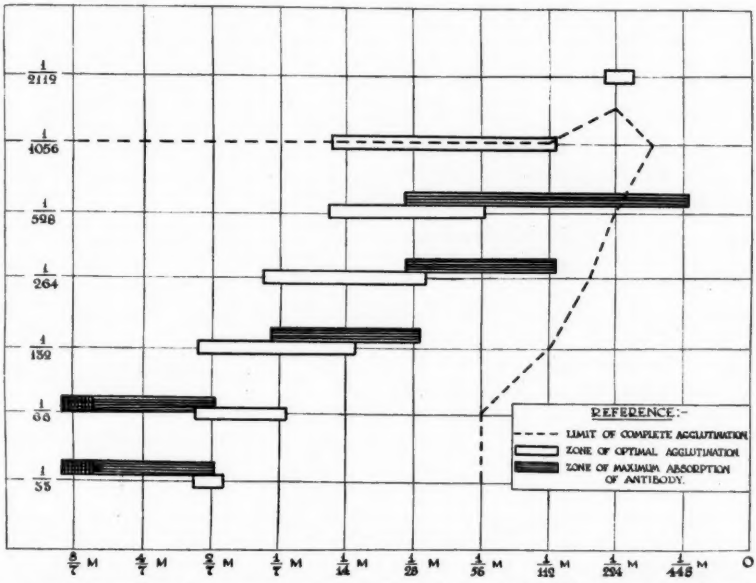

FIG. 5.—Agglutination of a constant mass of *Bact. dysenteriæ* Z (600 million organisms in 1 c.c.) by various dilutions of the homologous immune serum (both dialysed salt-free) at various salt concentrations, to show the relation of the *salt optimum* to the serum and salt limits (lowest concentrations) of agglutination. The figures have the same significance as in Fig. 1. The darker shading marking the zone of maximum absorption from 1/33 and 1/66 serum dilutions indicates that absorption was slightly greater at 8/7 *M* than at 4/7 or 2/7 *M* salt.

cohesive" effect of the strong salt on the stage of *particulation*. The anti-cohesive action of salt in preventing agglutination has been discussed by Northrop and De Kruif (1922), and Marrack (1934), dealing with the "second stage of the antigen-antibody reaction", quotes several observations of the inhibitory effects of high salt concentrations on precipitin and agglutinin reactions. The inhibitory effect of the 16/7 *M* salt is manifest only at low serum dilutions and at the serum end-titres, and the probable explanation of this is supplied by Duncan's observation, already referred to, that the cohesion of the particles is greatest at the antibody optimum (in this case at serum dilution 1/512), and diminishes progressively on both sides away from this point,

particularly in the direction of antibody excess. The anti-cohesive effect of the salt should be greatest where the cohesive tendency is least.

The relation of the salt optimum to the serum end titres and the low salt limits of complete agglutination is shown in Fig. 5. The general outline of agglutination is similar to that in Fig. 4, except that owing to the reduced antibody content of the dialysed serum the lower salt limit of agglutination cannot be traced beyond 1/56 *M* salt and there is no *complete* inhibition of agglutination by strong salt.

Optimum agglutination and maximum antibody absorption will be seen to follow generally the same course; the disparity between them at serum dilutions 1/66 and 1/33 is due to retardation of agglutination by the strong salt, although antibody absorption is at its maximum. The steep fall in the maximum absorption zone, from 8/7–2/7 *M* to 1/7–1/14 *M* salt, between serum dilutions 1/66 and 1/132 may be related to Duncan's (1934) observation that antibody absorption increases sharply in the presence of 1/7 *M* salt at the antibody optimum (in this case corresponding with a serum dilution of 1/96).

The influence of the salt optimum on the serum agglutination end-point curve in the H form of agglutination is clearly shown in Fig. 3, but this influence is less evident in the O form of agglutination, illustrated in Fig. 5, in which the salt optimum moves to a lower concentration progressively with the dilution of the serum until eventually it coincides, at 1/224 *M* salt, with the serum end titre of agglutination, causing a slight rise in the latter. However, the relation of optimal agglutination to the line of the lowest effective salt limits of agglutination seems definite in Fig. 5.

Dissociation of O antibody-antigen.—O antibody is dissociated from organisms strongly sensitized at *high* serum and salt concentrations, when they are dispersed in weaker salt solution or in distilled water. Therefore O antibody may be dissociated from flagellated bacteria at a salt concentration which is optimal for absorption of H antibody, and, *vice versa*, dissociation of the H antibody can be effected at strong salt concentrations which are optimal for O antibody absorption.

SUMMARY AND CONCLUSIONS.

The antibody, antigen and salt optima giving the highest velocity of agglutination have been interpreted as follows: The *antibody optimum* determines the most effective antibody sensitization of the bacteria and, in the O forms of agglutination studied, it resulted in that antibody-antigen compound which had the highest cohesive properties, as judged by the resistance of the flocculi to dispersion by mechanical force. It may be said, therefore, to affect the *quality* (firmness) of agglutination.

The *antigen optimum* depends on the highest concentration of adequately sensitized bacteria, and its effect is chiefly on the stage of particulation.

The *salt optimum* determines the maximum combination of antibody with antigen, and thus influences the *quantity* of agglutination measured by the highest effective serum titre.

Antibody-antigen combination reaches its maximum at the salt optimum,

and it diminishes progressively as the salt concentration deviates in either direction away from this optimum. The salt effects on the antibody-antigen reaction are reversible, so that dissociation of antibody from the sensitized bacteria occurs when the salt concentration is altered from that at which they were sensitized to one more remote, in either direction, from the salt optimum; and additional antibody can be absorbed by the sensitized organisms if the alteration brings the salt concentration nearer to the optimum.

The optimal salt concentration for the H form of agglutination is relatively low, and at high concentrations of the serum it tends to fall still lower as the *suspension*/serum ratio falls.

The optimal salt concentration for the O form of agglutination is high in the presence of high *serum*/suspension ratios and it falls progressively with the fall in this ratio.

A method of obtaining relatively pure H or O antibody by dissociation from sensitized bacteria is suggested.

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THE TYPE OF *CL. WELCHII* IN HUMAN FÆCES, WITH SPECIAL REFERENCE TO PERNICIOUS ANÆMIA.

GRIZEL R. BORTHWICK AND J. D. ALLAN GRAY.

From the Preventive Medicine Department, University of Bristol.

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SINCE the original demonstration of the exotoxin of *Cl. welchii* by Bull and Pritchett (1917) considerable attention has been focused on the toxins of the sporing anaërobic bacilli. Toxin-antitoxin neutralization experiments made in relation to classical *Cl. welchii*, lamb dysentery bacilli and *Cl. paludis* have shown that these organisms, though allied, differ greatly in the antigenic character of their toxins (Dalling, 1926; Weinberg and Ginsbourg, 1927; Mason, Ross and Dalling, 1931; Montgomerie and Dalling, 1933; Montgomerie and Rowlands, 1933; Glenny, Barr, Llewellyn-Jones, Dalling and Ross, 1933).

An outstanding contribution to the knowledge of the subject was made by Wilsdon (1931), who used these differences to classify the strains of *Cl. welchii* as types A, B, C and D. In addition he distinguished three toxic constituents, W, X and Z, of which W was produced by all four types, X by types B and D only and Z by types B and C only. Strains of type A were the classical types originally described as the ætiological agent of gas gangrene. Those of type B were found in lamb dysentery. Type C corresponded to *Cl. paludis*, while type D comprised *Cl. ovitoxicus* (Wilsdon, 1933) and certain strains of animal origin. Further differences between the types in their physiological action on perfused rabbit heart have recently been described (Nicholson, 1934–1935).

It was shown by one of us (Borthwick, 1935) that in experimental infections by different types, protection by monovalent sera usually depended upon the ability of the serum to neutralize the homologous toxin. The outstanding exception to this general rule was the power of the commercial *Cl. welchii* sera and sera prepared against type A toxin to protect against infection by organisms not only of type A, but also of type D, although the protecting sera were able to neutralize type A toxin only. That the inability of the commercial antisera to protect against infections of types B and C may be of great practical importance has been stressed elsewhere (Borthwick, 1935); for although type A is the recognized causal agent of gas gangrene in man, there does not appear to be any theoretical reason why the other types, which all contain some of the W constituent, should not also produce disease in man.

In 1928 Davidson demonstrated in pernicious anæmia a great increase in the organismal content of the bowel, especially of *Cl. welchii*. He ascribed ætiological significance to this finding, but, as judged by the hæmolytic and saccharolytic tests, he did not obtain evidence that any special strain of *Cl. welchii* was associated with pernicious anæmia. The purpose of the work here described was to apply the more recent knowledge of the mutliple antigenic structure of the toxins of *Cl. welchii*, in an attempt to ascertain whether the strains in the gastro-intestinal tract in pernicious anæmia differ materially from the type normally present in the human alimentary canal, or from the classical type A uniformly associated with gas gangrene in the human species.

EXPERIMENTAL PROCEDURE.

Sources of the strains of Cl. welchii *used.*—Known type strains required for comparative purposes were obtained from Dr. P. Bruce White (type A) and Dr. R. A. O'Brien (types B, C and D). In this paper these will be referred to as "standard" type strains, and the toxins and antitoxins prepared from them will be known as "standard" toxins and "standard" antitoxins, it being understood that the word "standard" has no quantitative meaning.

Specimens of healthy stools were obtained from an inmate of a mental hospital, a public assistance committee hospital patient and three laboratory workers. It was found that *Cl. welchii* could not be isolated from the stools of certain healthy individuals.

Specimens of the fæces of patients suffering from pernicious anæmia were obtained from Dr. Phillips, Superintendent of Southmead Hospital.

Patient 1 : Male, 57 years. In third relapse. Subacute combined degeneration of the cord ; gastrectomy five years ago. Blood condition stationary and will not respond to further treatment. R.B.C. 3,500,000.

Patient 2 : Female, 68 years. Typical primary anæmia. R.B.C. 1,930,000. Colour index 1·4.

Patient 3 : Female, 62 years. R.B.C. 2,870,000. Colour index 1·3. Died two months later from subacute combined degeneration of the cord.

Patient 4 : Female, 41 years. R.B.C. 945,000. Colour index 1·8.

Patient 5 : Female, 73 years. R.B.C. 1,100,000. Colour index 1·6.

Isolation of strains from fæces.—An emulsion of the fæces in saline was exposed to a temperature of 60° C. for half an hour to destroy non-sporing organisms. Tubes of veal broth were then inoculated with the emulsion and incubated for 24 hours anaërobically in a McIntosh and Fildes's jar. Subcultures on plates of dextrose blood-agar (Zeissler, 1930) were incubated anaërobically for 48 hours and typical colonies were subcultured anaërobically in veal broth. The organisms were then identified as *Cl. welchii* by morphological appearances, ability to liquefy gelatin, and power to ferment glucose, lactose, saccharose and maltose. The cultures were maintained in bullock's heart medium.

Preparation of toxin.—An inoculum of at least 10 c.c. from a culture in bullock's heart medium was introduced into a flask containing 500 c.c. at 37° C. of " Bouillon V.F." (Borthwick, 1935). After anaërobic incubation at 37° C. for a varying period the culture was filtered through wood-pulp and then through a Seitz filter fitted with a sterilizing disc. The toxin was concentrated by precipitation with ammonium sulphate, dried and ground to powder. In the dry state the toxins of all four types maintain their potency for very long periods, whereas according to Wilsdon (1931), those of types A and B are unstable in solution at room temperature. Throughout the work, therefore, toxins were measured by weight and not by volume.

Apart from the fluctuations in the toxin-producing power of individual strains, it was found that the period of incubation for optimum toxin-production varies with the different types. Thus, for the preparation of toxins of types A, B and C the incubation should not be prolonged beyond 24 hours, whereas for type D the optimal concentration is obtained after three days. (*cf.* Wilsdon, 1931). Various dilutions in saline were made of the toxins produced by the strains isolated from the cases of pernicious anæmia, and doses of 0·5 c.c. of the resulting solutions were administered intravenously to mice (see Table I).

TABLE I.—*Effect of Duration of Incubation of Strains from Cases of Pernicious Anæmia on the Production of Toxin.*

Toxin.	Average lethal dose in g. for mice after incubation of the " Bouillon V.F." culture for—		
	1 day.	2 days.	3 days.
P.A. 1	0·0010	0·005	0·010
P.A. 2	0·0020	0·010	Non-lethal
P.A. 3	0·0100	Non-lethal	,,
P.A. 4	0·0050	0·001	0·005
P.A. 5	0·0005	0·005	0·010

It will be seen that for each of these strains the amount of toxin produced after incubation for one day was greater than when the incubation was prolonged to two or three days. Actually the optimum period lay between 18 and 24 hours. This suggested that the strains isolated were not of the D type. The unit of toxin measurement adopted was the average lethal dose (A.L.D.). This was the minimal amount of toxin which killed within three days all of three mice after intravenous inoculation.

Preparation of antitoxins.—Antitoxins were prepared from the standard toxins and from the toxins of the strains isolated from the cases of pernicious anæmia. Formol-toxoids were made by incubation of the toxins with 0·1 p.c. formalin for four days at 37° C. Although the conversion of the toxin was probably not complete, the solution was detoxified sufficiently to be used for the immunization of rabbits. Weekly intravenous injections first of the toxoids and later of the toxins were administered over a period of from eight to ten weeks, and the value of the sera assessed by their powers to protect mice from doses of the homologous toxins of which the A.L.Ds. were known. The toxin-antitoxin mixtures were allowed to stand for one hour at room temperature before intravenous inoculation into the mice, and survival of the mice for three days was accepted as a proof of the protective powers of the antisera. Table II gives the results of these neutralization experiments for the standard toxins and the homologous antitoxins.

TABLE II.—*Neutralization of Standard Toxins by Monovalent Standard Type Antitoxins.*

Type.	Toxin A.L.D. (g.).	Amount (g. in 0·25 c.c. saline).	Neutralizing doses of antitoxin in 0·25 c.c. saline.			
			A.	B.	C.	D.
A	0·00100	0·00500	Undiluted	..	..	..
B	0·00001	0·00005	..	1/3125	..	..
C	0·00001	0·00005	..	..	1/625	..
D	0·00050	0·00100	..	..	..	1/25

NEUTRALIZATION EXPERIMENTS.

All the toxin-antitoxin neutralization experiments described in this paper were carried out on mice in the way just described for assessing the protective powers of the antitoxins.

Toxins of the Strains Isolated from the Cases of Pernicious Anæmia.

These were tested by neutralization with the standard type antitoxins (see Table III). All five toxins investigated were of the A type; for type A toxin is, firstly, the only toxin to be neutralized by type A antitoxin and, secondly, the only toxin to be neutralized by all of the four antitoxins (Wilsdon, 1931). Both these conditions were fulfilled by each of the five toxins. It is noteworthy that 5 A.L.D. of the first toxin (P.A. 1 in Table III) required

0·25 c.c. of undiluted standard type A antitoxin, while standard types C and D antitoxins could be much diluted and still neutralize a similar amount of the same toxin. Table II shows, however, that 5 A.L.D. of standard type A toxin likewise required for its neutralization 0·25 c.c. of undiluted homologous antitoxin. Tables II and III show further that, with one exception, more antitoxin of types B, C and D was required for neutralization of the toxins derived from pernicious anæmia strains than for neutralization of the homologous standard toxins.

TABLE III.—*Neutralization of Toxins of the Strains from Pernicious Anæmia by Monovalent Standard Type Antitoxins.*

Number.	Toxin A.L.D. (g.).	Amount used (g. in 0·25 c.c. saline).	Neutralizing doses of antitoxin in 0·25 c.c. saline.			
			A.	B.	C.	D.
P.A. 1	0·0010	0·0050	Undiluted	N.N.	1/125	1/25
		0·0025		1/25		
P.A. 2	0·0020	0·0100	,,	N.N.	1/100	Undiluted
		0·0050		1/25		
P.A. 3	0·0100	0·0500	1/5	1/5	1/50	,,
P.A. 4	0·0050	0·0100	1/5	1/25	1/250	,,
P.A. 5	0·0005	0·0010	1/5	1/25	1/25	,,

N.N. indicates " no neutralization ".

TABLE IV.—*Neutralization of Toxins of the Strains from Pernicious Anæmia by Antitoxins Prepared from them.*

Number.	Toxin A.L.D. (g.).	Amount used (g. in 0·25 c.c. saline).	Neutralizing doses of antitoxin in 0·25 c.c. saline.		
			P.A. 1.	P.A. 2.	P.A. 4.
P.A. 1	0·001	0·005	1/25	..	..
P.A. 2	0·002	0·010	..	1/15	..
P.A. 4	0·005	0·025	..	..	1/25

TABLE V.—*Neutralization of Standard Toxins by Antitoxins Prepared against the Toxins of the Strains from Pernicious Anæmia.*

Type.	Toxin A.L.D. (g.).	Amount used (g. in 0·25 c.c. saline).	Neutralizing doses of antitoxin in 0·25 c.c. saline.		
			P.A. 1.	P.A. 2.	P.A. 4.
A	0·00100	0·005000	1/25	1/25	1/5
B	0·00001	0·000050	N.N.	N.N.	N.N.
		0·000025	,,	,,	,,
C	0·00001	0·000050	,,	,,	,,
		0·000025	,,	,,	,,
D	0·00050	0·002500	,,	,,	,,
		0·001250	,,	,,	,,

N.N. indicates " no neutralization ".

Antitoxins were prepared for three of the toxins of the pernicious anæmia cases, viz. P.A. 1, P.A. 2 and P.A. 4, and tested by neutralization experiments against the toxins which had been used for their preparation. They were also used in neutralization experiments with the standard toxins (see Tables IV and V). Of the four standard toxins A, B, C and D, that of type A alone was neutralized. Even 2·5 A.L.D. of the standard toxins of types B, C and D were not neutralized by the undiluted antitoxins. It is of interest to note that the amounts of the antitoxin P.A. 1 required for the neutralization of 5 A.L.D. of the standard type A toxin was identical with the amount required for 5 A.L.D. of the toxin P.A. 1 from which the antiserum was prepared (see Tables IV and V). In the case of the antitoxin P.A. 4 a dilution of 1/5 was required for neutralization of 5 A.L.D. of the standard type A toxin, and a dilution of 1/25 for 5 A.L.D. of the homologous toxin P.A. 4.

Toxins of the Strains from the Fæces of Healthy Individuals.

Five toxins were prepared and tested with the standard antitoxins (see Table VI). The most noteworthy result was that two strains (N2 and N5), if toxic at all, were so weakly toxic that 0·01 g. failed to kill mice. The other three toxins (N1, N3 and N4) were obviously of the A type, as each of them could be neutralized by each of the four standard type antitoxins. As 5 A.L.D. of the first toxin (N1) were neutralized by 0·25 c.c. of a 1/25 dilution of the standard type A antitoxin, while 0·25 c.c. of the same antitoxin undiluted was required to neutralize 5 A.L.D. of the standard toxin (see Table II), it would appear that the toxin N1 was less toxic by about 25 times than the standard type A toxin. In spite of this the A.L.Ds. in grammes of the toxin N1 and the standard toxin were respectively 0·0005 and 0·001. This discrepancy cannot be explained by deterioration of the antitoxin, as the experiments recorded in Table VI were performed several months after those of Table II. Similarly two A.L.Ds. of toxins N3 and N4 were neutralized by the standard type A antitoxin in very high dilution.

TABLE VI.—*Neutralization of Toxins of Strains from Healthy Individuals by Standard Type Antitoxins.*

Number.	Toxin A.L.D. (g.).	Amount used (g. in 0·25 c.c. saline).	Neutralizing doses of antitoxin in 0·25 c.c. saline.			
			A.	B.	C.	D.
N1	0·0005	0·0025	1/25	1/125	1/25	1/5
N2	"Atoxic" (0·01 failed to kill)					
N3	0·0100	0·0200	1/3125	1/625	1/25	1/125
N4	0·0100	0·0200	1/3125	1/25	1/5	1/125
N5	"Atoxic" (0·01 failed to kill)					

Reference to Table III will show that, compared with the toxins of the strains from the cases of pernicious anæmia, the toxins of the three strains

from the fæces of healthy individuals (N1, N3 and N4) were more readily neutralized with standard type B antitoxin.

SUMMARY.

All of five strains of *Cl. welchii* isolated from the fæces of patients suffering from pernicious anæmia produced toxins of the A type, as shown by the short period of incubation required to produce the optimal concentration of toxin and the neutralization of the toxins by any of the four standard type antitoxins.

Of five strains from the fæces of healthy individuals, two were either completely or practically non-toxic, and the other three strains produced toxins of the A type.

Differences between the toxins of the strains from the two sources could therefore not be elicited other than in the degree of toxicity. Toxin, when produced in amounts suitable for testing, was consistently of type A. Evidence therefore is still lacking that man harbours *Cl. welchii* of types other than type A.

We desire to thank Drs. P. Bruce White and R. A. O'Brien for the standard cultures, and Dr. P. Phillips for the fæces of the patients suffering from pernicious anæmia.

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ON THE EFFECT OF 1 : 2 : 5 : 6-DIBENZANTHRACENE ON SPONTANEOUS MOUSE TUMOURS.

F. C. PYBUS AND E. W. MILLER.

From the J. H. Burn Research Laboratory, Royal Victoria Infirmary, Newcastle upon Tyne.

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FOR many years the apparent reversible action of X-rays and radium in producing and destroying neoplastic cells has impressed clinical and research workers. The production of epithelioma by tar suggested the possibility of its destruction by that substance, but tar has certain toxic effects, and it seemed probable that any action would consist of gross destruction of cells and would be non-selective. With the discovery of the pure carcinogenic hydrocarbons this objection was removed, and it is now possible to study the effect of these substances *in vivo* and *in vitro*, to test whether they have a reversible action analogous to that of radium and X-rays.

A recent paper by Haddow (1935) announced a striking effect of carcinogenic hydrocarbons on the growth of the Jensen rat sarcoma. The tumours, in hosts which were receiving daily intraperitoneal injections of an aqueous colloidal solution, grew very slowly compared with the control tumours. A massive dose of these substances in sesame oil had the same result (Haddow, 1936). This effect could be obtained with a few non-carcinogenic compounds. This valuable experiment demonstrates an apparently specific action on actively growing cells, unaccompanied by any general toxic effect, but it has certain limitations.

A transplanted tumour differs from the spontaneous in being composed of cells which are essentially foreign to the host, and in being an encapsuled growth in which all the cells are fully neoplastic. Consequently it is a much easier target for therapeutic attacks than a spontaneous tumour. The latter offers a different problem, since it consists of the animal's own cells, in all stages of hyperplasia, innocent and malignant, and it is consequently more difficult for any substance to act selectively on it.

In most of Haddow's experiments treatment was begun on the day of implantation, but in some of the later ones (1936) treatment began after an interval of 14 days. This prevented much further growth, but during the 14 to 20 days of the experiment (the animals being killed and the tumours weighed, 14 to 20 days after injection) there did not appear to be any actual decrease in size of the tumours. Growth was largely inhibited, but regression did not occur. The question arises whether the tumours would eventually have been absorbed completely, or commenced growing again, had the treatment been continued for a longer period.

It would seem to be essential also to discover the final effect on the

organism ; a large dose of a foreign substance might well prove toxic, affecting the kidneys, liver, etc., and the possible neoplastic action on the body generally must not be overlooked. In this connection it is as well to emphasize our lack of knowledge of the ultimate fate of carcinogenic substances after injection. Tumours appear in animals from which all trace of the causative agent has vanished. Investigations into this question are proceeding in various centres (British Empire Cancer Campaign, 1936). It would seem to be necessary to keep the treated animals for as long as possible, in order that the full effects of the substance may be noted.

If carcinogenic substances have a specific action on malignant cells, they might also be expected to pick out hyperplastic tissues. It would be important to test this on a strain of mice with a high incidence of mammary tumours, where hyperplastic and precancerous tissues must be frequent, to discover whether the substances could act as a prophylactic.

Since we considered it important to test the action of carcinogenic substances on spontaneous tumours, and were in the fortunate position of having a supply of spontaneous mammary tumours in mice, the following experiments with 1 : 2 : 5 : 6-dibenzanthracene were carried out. Our material was provided mainly by the Simpson strain, now in its fifteenth inbred generation in this laboratory, and giving practically 100 p.c. incidence of mammary tumours. Tumours occur in mice from the age of 6 months, and from that age until 15 months they are in good condition and there should be sufficient time to note the general effect of any treatment.

A histological examination of more than 500 mammary tumours has provided much experience of the many varieties which occur, and the necessary control material for these experiments (Pybus and Miller, 1936). Further material was furnished by two hybrid strains, and by the Edinburgh strain, which has a very low incidence of mammary carcinoma. Leukæmia occurs in all the strains, and sarcoma in the Simpson strain ; several cases of these were also treated.

EXPERIMENTAL METHODS.

In these experiments only 1 : 2 : 5 : 6-dibenzanthracene was used. It was given to tumour-bearing mice by two methods—injection and ingestion.

1. *Drinking.*—The colloidal solution, prepared by Boyland's (1932) method, was given instead of water for a month. An accurate estimate of the amount drunk by each mouse was not possible, but in the course of the experiment quantities varying from 29 to 45 mg. must have been absorbed.

2. *Feeding.*—The solid substance was mixed carefully with the normal food, 7·5 mg. being given to each mouse at each feed. The maximum total dose was 75 mg.

3. *Injection.*—(A) In *colloidal* form : The dose was worked out on the basis of body-weight of a mouse compared with a rat, following Haddow's technique (1935), and 0·5 c.c. (0·15 mg.) was the standard injection. The total dose varied from 1 to 10 mg. One series of tumours was treated by subcutaneous injection, but when it was found that this left a deposit of dibenzanthracene at the site of injection, the intraperitoneal method was substituted,

and injections were given daily or less frequently over a period of weeks. (B) *Suspension* in olive oil or gum saline, to test the effects of massive doses: Suspensions were prepared, in olive oil, of 15 mg. and 10 mg. per 0·5 c.c., and in gum saline of 15 mg., 10 mg., 7·5 mg. and 5 mg. per 0·5 c.c.; 0·5 c.c. was injected intraperitoneally into each mouse, either once only, or once a week for 2 or 3 weeks.

Owing to the size of the particles in suspension, it was not considered safe to attempt intravenous injection.

The tumours were charted every 7 or 14 days.

CONTROL EXPERIMENT.

Although we had a reasonably accurate knowledge of the varied rate of growth of untreated tumours, 11 mice were set aside as controls and the growth of their tumours recorded. Fig. 1 represents the growth of the

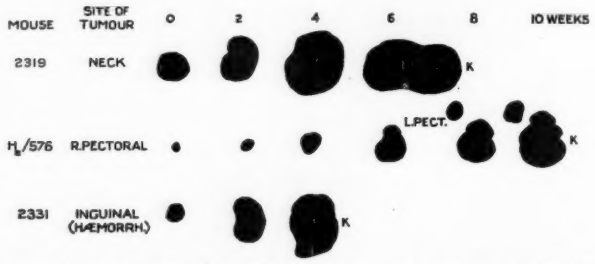

FIG. 1.—The growth rate of normal untreated spontaneous mammary tumours in 3 mice, controls for the 1 : 2 : 5 : 6-dibenzanthracene treatment. (Tumours charted life-size and reduced to 2/5 linear in reproduction; growth recorded every fortnight.)

tumours in 3 of these mice. Six mice were killed after periods ranging from 12 days to 10 weeks, on account of the size of their tumours, and the remaining 5 (in which the tumours had all grown rapidly, but were still of reasonable size after 8 weeks) were each injected with 5 mg. dibenzanthracene in gum saline (see below).

DRINKING EXPERIMENT.

Of the 6 mice in this series, 2 were killed after 19 and 23 days, and the rest after a month, all the tumours growing steadily to large size, as in the controls, with no signs of even temporary inhibition. At post-mortem examination the tumours were healthy, and there was no visible trace of the substance in the animals.

FEEDING EXPERIMENT.

There was an interval of 1 week between each dose of 15 mg. (given in 2 feeds of 7·5 mg.). Nine mice were treated, and Fig. 2 shows the rate of growth of tumours in 3 mice. The rate resembled that of the control tumours, with no sign of inhibition, and all the animals were healthy when examined *post*

mortem. Two mice were killed after 3 weeks (45 mg.), and 5 after 7 weeks (75 mg.), while the remaining 2 received an injection of 5 mg. each during the eighth week, without effect.

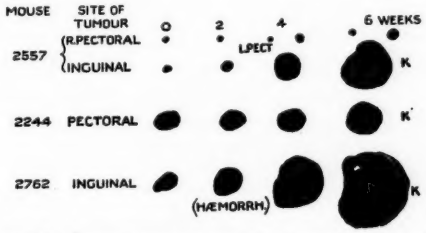

FIG. 2.—The rate of growth of spontaneous mammary tumours in 3 mice, each of which had been fed on 15 mg. dibenzanthracene weekly for 5 weeks.

INJECTION EXPERIMENTS.

Colloidal Solution.

Subcutaneous injections only.

Leukæmia.—Four mice were treated; 1 died almost immediately; the other 3 were killed after 9, 10 and 13 injections respectively, no improvement being apparent.

Mammary carcinoma.—The results in this series may be given in the form of a table:

	Number of mice.	Number of injections.	Number of hæmorrhagic tumours.	New tumours during treatment.
Treated	17	..	..	..
Died early	4	..	..	..
Steady growth of tumour	7	6–19	3	2
Neither growth nor regression of tumour	4	6, 8, 16	0	0
Temporary regression of tumour	2	10, 19	0	3

In the first mouse in which regression occurred, the tumour was no longer palpable after 12 days, but at the post-mortem, 33 days later, a thin plaque of carcinoma was present. The second mouse had 3 tumours situated in different glands. One regressed slightly during the first 4 weeks, the second neither grew nor regressed, but the third (hæmorrhagic) grew steadily. The mouse was killed 37 days after treatment began, owing to the renewed or continued growth of these tumours and the appearance of 3 new tumours. Every mouse in this series had a subcutaneous deposit of dibenzanthracene at the site of injection, and very little could have been absorbed.

Subcutaneous injections, followed after an interval of 16 *days by a course of intraperitoneal injections* (2 *or* 3 *per week*).

	Number of mice.	Number of injections.	Duration of treatment (days).	Number of hæmorrhagic tumours.	Number of new tumours.
Treated	11	..	..	..	..
Died early	3	..	..	..	..
Rapid growth of tumour	5	10–19s + 5–20p	49–55	3	2
Neither growth nor regression of tumour	1	10s + 3p	49	0	0
Temporary regression of tumour	2	15s + 45p	126	2	0
		17s + 13p	98		

s = subcutaneous. p = intraperitoneal.

In Fig. 3, in Mouse 2440, slow growth was followed by a very rapid increase in size; intraperitoneal injections, begun when the growth-rate changed, had no effect. In Mice E/514 and 1555 temporary regression occurred.

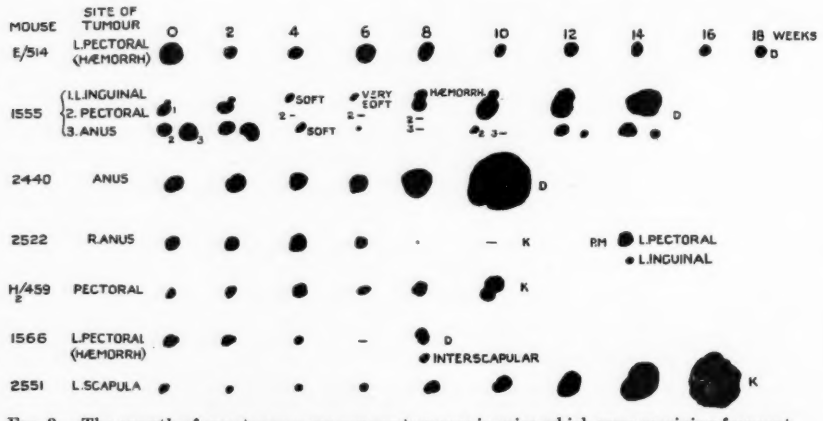

FIG. 3.—The growth of spontaneous mammary tumours in mice which were receiving frequent intraperitoneal injections of colloidal 1 : 2 : 5 : 6-dibenzanthracene. (In the first 2 cases the subcutaneous method of treatment preceded the intraperitoneal.)

In Mouse E/514 the subcutaneous injections were spread over 4 weeks; intraperitoneal injections began during the sixth week, when the tumour had increased in size. When the mouse died, it had received a total amount of 9 mg. dibenzanthracene. Histological examination of the tumour was impossible owing to decomposition.

In Mouse 1555, all 3 tumours regressed during the 4 weeks of subcutaneous inoculations. Intraperitoneal injections were started during the eighth week, when the inguinal tumour had grown considerably. During the twelfth

week an intraperitoneal injection of 15 mg. in 0·5 c.c. gum saline was given, without effect. Histologically, the inguinal tumour was mainly acinous, degenerate in parts, but actively growing in other regions; the pectoral tumour, mainly acinous and actively growing, contained more connective tissue than usual; the third tumour was of the scirrhous type.

Intraperitoneal injections only.

Sarcoma.—Two mice, with sarcoma of humerus and tibia respectively, showed no improvement after 15 and 32 injections, nor had a subsequent massive dose of 15 mg. any effect; both sarcomata grew rapidly and metastasized, and the animals were killed after 2 months.

Leukæmia.

	Number of mice.	Number of injections.	Duration of treatment.
Treated	8	..	..
Died early	1	..	..
No improvement	4	7–17	3–5 weeks
Temporary improvement	3	20, 29, 34	6, 8, 10 weeks

The temporary improvement consisted of a reduction in the size of the spleen or superficial lymphatic glands, but the symptoms in each case became more pronounced and death followed rapidly.

Mammary carcinoma.

	Number of mice.	Number of injections.	Duration of treatment (weeks).	Number of hæmorrhagic tumours.
Treated	12	..	..	..
Steady growth of tumour	4	11, 13, 20, 32	4, 5, 6, 9	2
Temporary inhibition of growth of tumour	6	29, 40, 43, 44, 55, 30	9, 13, 12, 14, 17, 8	0
Temporary regression of tumour	2	39, 33	10, 9	1

In the cases of temporary inhibition the tumour did not alter appreciably in size for 6 to 9 weeks, and a slight diminution in size was noticed occasionally. Mouse 2551 (Fig. 3) is an example of this. When growth was resumed it was usually rapid, but in 2 cases the tumours continued to grow slowly. Four of the tumours were of the loose acinous type, one of these being partly scirrhous; the other 2 were alveolar and the cells were dividing actively. Early carcinomatous changes were found in the other mammary glands of 2 mice.

The history of the regressing tumours is shown in Fig. 3, Mice 2522 and 1566. The former was not palpable after 10 weeks' treatment, and inoculations ceased. When the animal was killed 2 weeks later, it was found to have 2 new tumours, but of the original anal tumour there was no trace, and only fibrosed ducts were visible in microscope sections. The new tumours were of the acinous-alveolar type, but in one there was a rapidly-growing area of cellular connective tissue. In Mouse 1566 the tumour was not palpable

after 6 weeks' treatment. Inoculations were continued for a further 3 weeks, with the result shown. Both tumours consisted of large degenerate areas, and the pectoral tumour was scirrhous in part; much connective tissue was present in the second tumour, which was, however, mainly alveolar in type.

Later experiment.—As all the experiments (to be described) in which the massive doses were used resulted in the death of the mice from toxic effects, and in view of the partial success of the above treatment, a further series of 10 tumour mice were injected daily with 0·5 c.c. of the colloidal solution, intraperitoneally. Eight of the 10 tumours grew progressively, some even rapidly, but 2 disappeared completely after 16 inoculations each. The daily injections were continued in these 2 mice; neither tumour recurred, and when the animals were killed on account of ill-health after 25 and 45 injections respectively, no trace of a tumour was seen at either autopsy. After 8 weeks' treatment most of the mice were killed, and all had moderate ascites and pleural effusion. There was a complete absence of particles of dibenzanthracene from the peritoneum.

Intraperitoneal injections, followed by massive doses.

After 2 months' treatment with the colloidal solution, 5 mice in which the tumours were growing rapidly received an intraperitoneal injection of 15 mg. in 0·5 c.c. gum saline, while another 5 in which growth was rather slower received this amount in fractionated doses (with 1 week's interval) of 7·5 mg.

The large dose of 15 mg. caused the deaths of all the mice from peritonitis.

	Number of mice.	Result.
Treated	5	..
Dead 1 day later	2	..
Dead 11 days later	1	Hæmorrhagic tumour much smaller.
Dead 14 days later	1	Slight regression of tumour.
Killed 18 days later	1	No improvement.

The injections of 7·5 mg. had no effect on the tumours. The previous history of one of these tumours is shown in Fig. 3; Mouse H_2/459 received 43 colloidal injections during 10 weeks. When the tumour doubled its size in a fortnight one injection of 7·5 mg. was given, but the mouse had to be killed a few days later on account of peritonitis. This tumour was of the scirrhous type, with a small healthy core, surrounded by a necrotic area. A new tumour, found at post-mortem, was of the rapidly growing acinous-alveolar type, accompanied by extensive neoplastic changes throughout the mammary gland.

Another mouse, 1854, had a tumour in the pectoral gland, which under colloidal treatment was becoming steadily smaller, while one in the inguinal region was growing slowly. After 8 weeks (28 injections), a third tumour appeared beside the ear, growing very rapidly. In spite of more frequent inoculations, at the tenth week (39 inoculations) a fourth tumour appeared close to the anus. Two injections of 7·5 mg. had no inhibitory effect on either of these rapidly growing tumours, and the animal was killed a week after the second injection. The histology of the regressing pectoral tumour was of

special interest; diffuse carcinoma was present in an abnormal and rich development of spindle-celled tissue. The other three tumours were healthy acinous growths.

In the tumours of 2 of the remaining 3 mice in this series the connective tissue was again well developed.

15 *mg. Suspension in* 0·5 *c.c. Olive Oil.*

Fourteen mice were treated, but 4 died soon after inoculation. In 9 mice the tumours were appreciably smaller 14 days after inoculation, while in the tenth the hæmorrhagic tumour had not altered in size; but a fortnight later the tumours in 3 mice had resumed growth, new tumours appearing in 2 of these, while in a fourth animal (with 2 pectoral tumours) one resumed growth while the other continued to regress. These 4 animals then each received 2 injections of 7·5 mg. in gum saline, without effect. In the remaining mice, as shown in Fig. 4, regression continued until 4 weeks after inoculation, but after that period growth was resumed in the tumours of the 4 surviving mice.

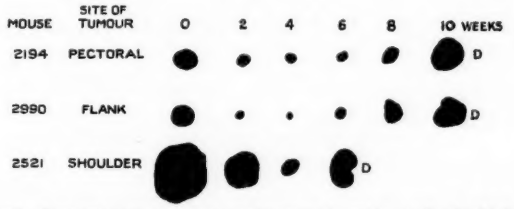

FIG. 4.—The growth of spontaneous mammary tumours in mice, each of which had received one intraperitoneal injection of 15 mg. dibenzanthracene in 0·5 c.c. olive oil.

The death-rate in this series was high, the majority of the mice dying of peritonitis, ascites and pneumonia quite early in the experiment. The results are thereby obscured. Thus in one mouse which died at 4 weeks the tumour had disappeared completely, but judging by the behaviour of the tumours in the surviving mice one cannot conclude that this was a cure.

10 *mg. Suspension in* 0·5 *c.c. Olive Oil.*

In an attempt to reduce the toxicity, the dose was reduced to 10 mg. given in 1 injection to each of 12 mice. Three died almost at once; 1 tumour, hæmorrhagic, grew slowly but steadily for 20 days, but the remaining 8 diminished. In 9 days, when the host died, one tumour had become less than half its original size, in spite of being hæmorrhagic. Fig. 5 shows the

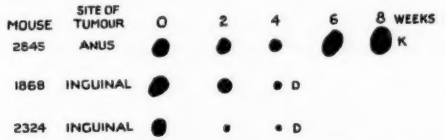

FIG. 5.—The growth of spontaneous mammary tumours in mice, each of which had received one intraperitoneal injection of 10 mg. dibenzanthracene in 0·5 c.c. olive oil.

typical behaviour of the tumours in those mice which survived. Only 4 mice were alive at 4 weeks, and at 5 weeks the tumours in the 2 remaining animals had resumed growth. In one of these a second nodule appeared during the seventh week. The death-rate was again very high in this series, from peritonitis, ascites and pneumonia.

15 *mg. Suspension in* 0·5 *c.c. Gum Saline.*

This suspension was used to treat 3 cases of leukæmia, and the 5 cases of mammary tumours mentioned above which had had previous injections with colloidal solution. The leukæmias showed no improvement, and peritonitis was as frequent as with the oil suspensions.

7·5 *mg. Suspension in* 0·5 *c.c. Gum Saline.*

In a further attempt to reduce the toxicity, the 15-mg. dose was split into two of 7·5 mg. given with 1 week's interval. Eleven mice were treated, but 4 (including 2 cases of leukæmia) died early in the experiment. One case of sarcoma showed no improvement and the tumour grew steadily and metastasized. Fig. 6 shows the type of behaviour encountered in 3 of the 6 cases

FIG. 6.—The growth of spontaneous mammary tumours in mice, each of which had received two intraperitoneal injections of 7·5 mg. dibenzanthracene in 0·5 c.c. gum saline.

of mammary carcinoma which survived. With the exception of Mouse $H_2/599$, all the surviving tumours were growing steadily at 5 weeks. An injection of 5 mg. in gum saline was given to each mouse, with no definite effect beyond accelerating death from peritonitis in all except $H_2/599$. This animal received another injection of 5 mg. during the seventh week, as the tumour reappeared and was growing, and this was followed by a dose of 12·5 mg. (in error) in the eighth week, with no effect on the tumour. The mouse lived for another 10 days. Histologically this was an acinous tumour, scirrhous in part.

5 *mg. Suspension in* 0·5 *c.c. Gum Saline.*

This was a final attempt to avoid the toxic effect, and 29 mice were treated, including 5 cases of leukæmia, 1 epithelioma (which died 3 days later), 3 sarcoma and 17 mammary carcinoma. Inoculations were given weekly for 2 or more weeks.

Leukæmia.—Three died immediately, and no improvement was noticed in the 2 survivors.

Sarcoma.—No improvement occurred.

Mammary carcinoma.—The results are given in the form of a table.

	Number of mice.	Number of injections.	Duration of life (weeks).
Progressive growth	6 { 5	3	5(3), 8, 4, 3
	6 { 1	4	
Size of tumour unaltered	3	3	3, 4, 5
Slight temporary regression	2 { 1	3	3
	2 { 1	1 + 1 of 2·5 mg.	4
Regression	6 { 1(2591)	2 + 2 of 2·5 mg.	7
	6 { 1(H_2/727)	2	6
	6 { 1	2	5
	6 { 1	3	4
	6 { 1(2195)	3	4
	6 { 1(H_2/464)	2 + 2 of 2·5 mg.	4

Fig. 7 shows the types of behaviour of some of these tumours. Progressive growth and the development of a new tumour are seen in Mice 2476 and E/588. The 2 tumours which showed slight temporary regression resumed

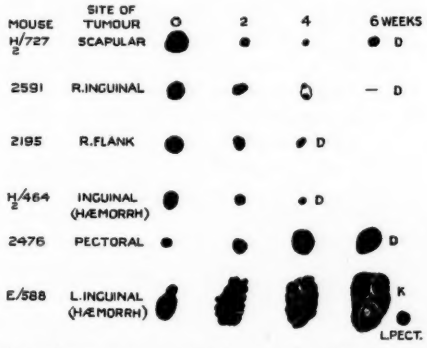

FIG. 7.—The growth of spontaneous mammary tumours in mice, each of which had received two or more intraperitoneal injections of 5 mg. dibenzanthracene in 0·5 c.c. gum saline.

growth after a week ; both were cystic. Four of the 6 cases of regression are shown in Fig. 7. In each case the value of the observation is obscured by the untimely death of the animal from ascites and peritonitis, but from Mouse H_2/727 it seems that growth would have been resumed after 4 or 5 weeks as in the other experiments ; in this mouse the tumour was a small but diffuse carcinoma, scirrhous with much fibrosis towards the centre, and healthy dividing cells at the edge.

The tumours of the 5 control mice, which, as mentioned above, were each inoculated twice with 5 mg., showed no improvement, and all died of ascites and peritonitis.

DISCUSSION OF RESULTS.

The results of these experiments support Haddow's work that 1 : 2 : 5 : 6-dibenzanthracene has an inhibiting effect on the growth of neoplastic cells, and

demonstrate that this effect applies also to spontaneous tumours. Sarcoma and leukæmia appear to remain unaffected by the treatment, but a proportion of mammary tumours regressed. This fact is of some importance, as no example of diminution in size or disappearance of an untreated spontaneous mammary tumour has been seen. The colloidal solution, injected either subcutaneously or intraperitoneally, was responsible for 8 regressions, partial or complete, in 60 mice, but in only 3 cases was there no reappearance of the tumour before death, and, in one of these, 2 new tumours were present in other sites at death.

There is some evidence that growth was inhibited to a greater or less degree in many cases ; for a number of weeks the tumour either did not increase in size at all (11 cases), or the rate of growth appeared to be much less than in the controls. But in 29 cases the tumours grew rapidly and did not respond to treatment.

The percentage of regressions was much greater, and the speed with which they occurred more striking in mice treated with massive doses in suspension. Only 1 tumour in each of the 15 mg. and 10 mg. series grew progressively ; all the rest (in those mice which survived long enough) had become very much smaller in 14 days, and remained small for another 14 or 21 days, but, at the end of that period, growth in the few survivors was resumed at a normal rate. The result with 7·5-mg. doses was less striking and only one tumour regressed temporarily. The 5-mg. injections were more effective, and 6 out of 17 tumours regressed ; in those animals which survived, however, growth was resumed after 4 or 5 weeks.

The effect of the treatment appears to be temporary only, and it is perhaps unfortunate that Haddow removed the treated transplanted tumours after a period of only from 18 to 24 days. As the above experiments show, if a tumour is regressing, the maximum effect is apparent just at that time, and it would have been important to know the further fate of the transplanted tumours for comparison.

In the limits of the work accomplished, it appears that the methods of exhibition of 1 : 2 : 5 : 6-dibenzanthracene are open to objection by their local action on the peritoneum and abdominal viscera, in causing ascites or gross peritoneal adhesions, and degeneration of the liver and kidneys. The massive doses especially were responsible for a very high mortality, and particles of solid dibenzanthracene were deposited on the peritoneum and remained there for many weeks. Methods of exhibition which succeeded with rats were therefore evidently not suitable for mice, which seem to be much more delicate.

Owing to the high death-rate, the original intention could not be carried out of keeping the successfully treated animals for a long period, to discover whether dibenzanthracene had any general carcinogenic effect.

The size of the tumour at the beginning of treatment, and more especially its type, are factors which probably influence its response. Massive doses, given to mice bearing large, steadily growing tumours which had received colloidal treatment, had no effect in most cases. Tumours of the hæmorrhagic cystic type were rarely checked in their growth, although three of the regressing tumours were of this type. When more than one tumour was present in a mouse, the individual tumour reaction was most noticeable, and one might regress steadily while the others grew rapidly.

Histology.—While it is a question to what extent the high percentage of temporary regressions was due mainly to the poor condition of the mice which had received massive doses, rather than to any action of the dibenzanthracene, a histological examination of the treated tumours suggests that they often contain an unusually rich development of connective tissue, and to such an extent was this so in two tumours (one regressing in Mouse 1854, and the other a new tumour appearing during treatment in Mouse 2522) that it almost resembled a sarcoma. Comparison of the amount of necrotic tissue present was unsatisfactory when so many of the tumours showed post-mortem changes ; in any case, necrotic areas are a feature of the majority of mammary tumours which reach any size. Nevertheless, a number of regressing or very slowly growing tumours appeared to be more generally necrotic than usual. Regression was often but not invariably associated with the development of scirrhous types or fibrosis, many regressing tumours being of the loose acinous type. In some the capsule was well defined, and in one case much thickened. When growth was resumed, alveolar and alveolar-papillary types were found.

SUMMARY.

When 60 mice bearing spontaneous mammary tumours were injected intraperitoneally with a colloidal solution of 1 : 2 : 5 : 6-dibenzanthracene, the tumours regressed partly or completely in 8 cases, and in 3 of these did not reappear before death ; but the formation of new tumours during treatment was not prevented. A temporary inhibition of growth was noticed in 11 cases. The maximum total dose was 10 mg.

Massive doses given in one injection of 15 mg. or 10 mg. in olive oil suspensions, or in two or more fractionated doses of 7·5 or 5 mg., caused a temporary regression of the tumour in 26 out of 61 cases, the effect reaching its maximum about the third and fourth weeks, but growth was resumed after that period. These large doses caused a very high mortality from ascites and gross peritoneal adhesions.

The treatment was without effect on cases of sarcoma and leukæmia.

Oral administration, as a drink, or by feeding the solid substance, had no effect on spontaneous mammary tumours.

Histologically, the treated tumours consisted of a higher proportion than normal of scirrhous and fibrosed types, and the connective tissue was well developed in many tumours.

It is concluded that 1 : 2 : 5 : 6-dibenzanthracene has an inhibiting effect on the growth of spontaneous mammary tumours, but that this effect is only temporary.

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VARIATIONS IN FOWL-PEST VIRUS.

R. D. MACKENZIE AND G. M. FINDLAY,

Wellcome Bureau of Scientific Research, London.

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THE observations of Andervont (1929) on the effects of intracerebral inoculation of herpes virus into species not ordinarily susceptible by intraperitoneal or subcutaneous inoculation, and the employment of this method by Theiler (1930) for the production of a neurotropic variant of the yellow fever virus, have provided subsequent workers with a convenient means of approach to many virus problems. Later it was shown by Findlay and Stern (1935), and independently by Penna (1936), that neurotropism in the yellow fever virus could be demonstrated in animal species which are highly susceptible to subcutaneous or intraperitoneal inoculation, if the viscera were previously protected by an injection of immune serum.

This method was taken advantage of and extended by Mackenzie and Findlay (1936), who demonstrated that a neurotropic variant of the virus of Rift Valley fever could also be formed by passage in the brains of mice, provided the viscera of these highly susceptible animals had previously been passively immunized. In view of the successful results that were obtained when this method was applied in the case of Rift Valley fever, it was decided that observations should be made on the behaviour of certain other viruses when exposed to similar experimental conditions. After some consideration fowl pest was selected for this purpose, since it can conveniently be maintained in fowls under laboratory conditions, and has in the past frequently shown evidence of neurotropic activity. Thanks to the kindness of Dr. T. M. Doyle, of the Ministry of Agriculture, we obtained a strain of this virus, and throughout this communication it will be referred to as the " Doyle strain ". It has been propagated in hens for many years, and was originally isolated from an outbreak in this country.

As far as the present paper is concerned it will not be necessary to include a detailed account of the work of the early investigators of fowl pest, since the results about to be described will deal mainly with such variations in the activity and character of the Doyle strain of virus as have resulted from the conditions to which it has been subjected. Hutyra and Marek (1926) and Todd and Rice (1930) give satisfactory summaries of the disease.

EXPERIMENTAL.

Preliminary.

In order both to obtain an adequate supply of infected material, and to test the activity of the virus, a portion of the infected fowl's liver, which had been supplied to us preserved in saline glycerine, was washed in sterile saline solution

and ground in a mortar. Normal saline solution was added so as to make approximately a 10 p.c. suspension. This was lightly centrifuged, and 0·25 c.c. of the supernatant fluid was inoculated intraperitoneally into two hens. Next day both birds seemed depressed, having lost their ordinary alertness and desire for food. After 48 hours they were obviously sick, their eyes being partially closed, and it was only with difficulty that they could stand. During the night they both died, and when examined no obvious naked-eye pathological lesion was seen.

Preparation of Immune Serum.

Before experimental work could begin, it was first of all necessary to prepare a supply of immune serum. This, it was originally intended, should be obtained from fowls which had previously been immunized by means of the phenol glycerine liver vaccine of Todd (1928). Unfortunately in this case, as in the experience of Purchase (1930), the method was not successful, and little or no evidence of immunity was noted. Since it thus seemed difficult to use fowls for this purpose, it was decided that the immune serum should be prepared in rabbits, which are either insusceptible, or only contract an inapparent form of the disease. Accordingly 2 rabbits were inoculated intraperitoneally with an emulsion made by grinding the liver of a fowl dying of fowl pest. Before injection the liver emulsion was lightly centrifuged, and only the supernatant fluid was used, one adult fowl's liver being considered sufficient for 2 rabbits. Three similar weekly injections were given, and in the 4th week the rabbits were bled, and their sera pooled.

Production of a Neurotropic Strain.

Since adult fowls were not very convenient for a series of intracerebral inoculations, and as it was difficult at the time to arrange for a supply of young chickens, it was decided that the experiment should be carried out in canaries. Before this was done, however, a few preliminary experiments had been performed which demonstrated that these birds were just as susceptible as fowls to the action of the virus, whether inoculated intracerebrally or intraperitoneally. Pigeons were not considered suitable for this purpose since they have frequently been found to be refractory to the disease. This lack of reaction has been considered by Doyle (1927, 1935) to differentiate between fowl pest and Newcastle disease—a condition to which they are very susceptible. The value of this method has been questioned by Manninger (1932, 1936), who considers that these diseases represent an ætiological entity.

In the canary.—For this purpose 2 canaries were given an intraperitoneal injection of 0·25 c.c. of fowl-pest immune rabbit serum, and then about 30 minutes later both birds were anæsthetized and inoculated intracerebrally with 0·03 c.c. of a 10 p.c. emulsion of fowl-pest infected hen's liver. Suitable culture controls were always made to ensure the bacteriological sterility of the inocula used in these experiments.

Some 24 hours later the canaries appeared to be unable to fly, and to have difficulty in balancing on the perches. Their feathers were puffed out and necks retracted, giving them the appearance of a ball of feathers. Later in the day

they could not remain on the perch, and by next morning both were dead. Their brains were removed, ground in a mortar with saline so as to make a 10 p.c. suspension, which was then lightly centrifuged, and 0·03 c.c. of the supernatant fluid was injected into the brains of 2 fresh canaries previously immunized as before. In this way subinoculation of the virus has been carried on more than 40 times, and it has always produced a reaction very similar to that described above. In fact the only difference that has been noted is a gradual increase in the pathogenicity of the virus, so that it now kills in about 24 hours.

In the mouse.—When the pathogenicity of the Doyle strain was being estimated in fowls and canaries, an attempt was made to infect white mice by intracerebral injection. Unfortunately only 6 mice were used at this time, and since none developed symptoms, the result was considered to be completely negative. From later knowledge of the disease, it appears certain that if a larger number of animals had been used, the infection would have been successfully transmitted to white mice at this stage. Evidence in support of this will be found later in this paper.

As the first attempt was unsuccessful, no further effort was made until the strain of fowl pest had been transmitted serially 28 times in immunized canaries, when another batch of 6 mice were inoculated with this 28th canary passage strain. Four to 6 days later 4 of them showed symptoms, such as trembling, circular movement and spasms, all suggestive of cerebral irritation. Further batches of mice were inoculated from these by cerebral passage, and they showed similar symptoms, along with a gradual shortening of the incubation period, so that at the 11th intracerebral mouse passage, 13 out of 30 inoculated mice died within 48 hours ; there was an eventual mortality of 100 p.c.

These results agree with those of Jansen and Neischulz (1933) and Neischulz and Bos (1934), who also obtained a similar mortality. It is not, however, easy to account for some of the results reported by Doerr, Seidenberg and Whitman (1931), who, having shown that the virus of fowl pest could be carried in the brains of white mice, attribute the subsequent death of these animals to the incidental inoculation with homologous brain substance. In support of this they state that in their experience the intracerebral inoculation of normal mouse brains into mice is frequently lethal. In connection with various experiments, we have had occasion to inoculate both normal and infected mouse brains into a large number of mice, and we have never noted a death that we could attribute to such a cause. Although these authors believed that they had excluded the possibility, it would seem that their " normal " mice may have been carriers of some other virus infection, such as lymphocytic meningitis, as described in America by Traub (1935), and by Findlay, Alcock and Stern (1936) in this country.

Comparative Pathogenicity in Mice of the Doyle Strain, and the Two Variants of the Fowl-pest Virus.

Since, as a result of the above procedure, we now possessed three " strains " of fowl pest, *i. e.* the Doyle or original strain, and the canary and mouse variants, it was decided that a comparison should be made regarding their relative

pathogenicity. As a matter of convenience it was arranged that this should be carried out in mice. For this purpose 10 p.c. suspensions of infected fowl's liver for the Doyle strain, and infected canary and mouse brains, representing the 35th and 11th passages respectively, were prepared in normal saline, and batches of 30 mice of approximately the same age and size were inoculated intracerebrally, intraperitoneally or intranasally with each of these suspensions. These mice were observed for 10 days, and all deaths occurring during this period were noted.

TABLE I.—*The Comparative Pathogenicity in Mice of the Doyle Strain of Fowl Pest and the Canary and Mouse Variants.*

	A.			B.		
	Original.	Canary.	Mouse.	O.D.	S.E.	$\frac{O.D.}{S.E.}$
I.P.	0	7	..	23·3	8·3	2·8
	0	..	24	80·0	12·7	6·3
	..	7	24	56·7	12·9	4·4
I.C.	6	24	..	60·0	12·9	4·7
	6	..	30	80·0	12·7	6·3
	..	24	30	20·0	7·8	2·6
I.N.	3	18	..	50·0	12·3	4·1
	3	..	28	83·3	12·9	6·5
	..	18	28	33·3	10·9	3·1

A. The figures indicate the number of mice out of 30 in each instance dying within 10 days as a result of inoculation with one of the strains of fowl pest by the route shown.
I.P. = Intraperitoneal route. Dose, 0·2 c.c.
I.C. = Intracerebral route. Dose, 0·03 c.c.
I.N. = Intranasal route. Dose, 2 drops in nostril.
B. The observed difference (O.D.) and standard error (S.E.) have been calculated as percentages.

The results of this experiment are shown in Table I, and there it will be seen that the Doyle strain is only slightly pathogenic for mice, even when inoculated by the intracerebral route, while the canary variant occupies an intermediate position between the Doyle strain and the mouse variant, which is now highly pathogenic for mice. To make the distinction more obvious, the observed difference and standard error have been calculated, and it will be found that in 7 out of 9 observations a significant result has been obtained.

It would appear to be a matter of interest that in such a comparatively short period it has been possible so to modify the Doyle strain of fowl pest as to result in the production of two characteristic variants, which differ greatly from their parent strain and from each other. This observation may help to explain the variability in the pathogenicity of the virus as reported by various authors, such as Plotz (1936), who found that only some strains of fowl pest will infect mice, Lépine (1936), who records a mortality of 40–50 p.c. in mice inoculated intracerebrally, and Jansen and Neischulz (1933), and Neischulz and Bos (1934), who obtained a mortality of 100 p.c. Recently Oerskov, Schmidt and Steenberg (1936) have experimented with two strains of fowl pest, one highly pathogenic for mice, and the other relatively feeble in this respect.

Distribution of the Mouse Variant of Fowl-pest Virus after Intraperitoneal Inoculation in the Mouse.

Since the mouse variant of the fowl-pest virus (Table I) has shown itself to be highly pathogenic for mice, even when inoculated by the intraperitoneal route, it seemed desirable that a few observations should be made on the distribution and localization of the virus after intraperitoneal inoculation. Mice were, therefore, injected intraperitoneally with 0·2 c.c. of a 10 p.c. emulsion of infected mouse brains of the 14th mouse passage. From amongst these, lots of 3 were killed at predetermined intervals, and certain of their organs and tissues were removed and pooled. Batches of 6 normal mice were then inoculated intracerebrally with suspensions of these tissues. As was shown by the fate of the latter mice (Table II), virus was present in large

TABLE II. — *Distribution in the Mouse of the Mouse Variant of the Fowl-pest Virus after Intraperitoneal Injection at Varying Intervals after Inoculation.*

	Blood.	Spleen.	Adrenal.	Brain.
3 hours . . .	0	6	0	0
1 day . . .	0	6	1	0
2 days . . .	0	6	0	0
3 ,, . . .	0	6	5	4
5 ,, . . .	0	3	6	6

Figures indicate the number of mice out of 6 dying within 10 days as a result of intracerebral inoculation with the pooled tissues of 3 mice killed at the periods stated.

amount in the spleen from the first observation at 3 hours until 3 days had elapsed, but subsequently there was a slight reduction in the amount present. On the other hand, the brain and adrenal did not show any degree of infection until the third day after intraperitoneal inoculation, and they were still highly infective on the 5th day. Throughout this series of observations the blood was constantly free from infection, which would seem to indicate that the spread of virus to the brain and adrenal had probably not been directly by way of the blood-stream.

Pathogenicity in Mice of Two Original Strains of Fowl Pest.

At this stage it was considered that the value of these experiments would be increased if they included a comparison between the Doyle strain and another original strain of fowl pest. Our thanks are due to Dr. C. Todd and Mr. I. A. Galloway, of the National Institute for Medical Research, who generously supplied us with a strain which was obtained about ten years ago from Prof. Doerr, in Switzerland, and which has been maintained in fowls in this country. With these two original strains of fowl pest, an experiment was carried out on very similar lines to that described above, and further batches of mice were inoculated by the intraperitoneal, the intracerebral and the intranasal routes. The results (Table III) show that no difference could be demonstrated by the action of these two strains in mice.

TABLE III.—*The Comparative Pathogenicity in Mice of Two Original Strains of Fowl-pest Virus.*

	Strains of fowl pest.	
	Doyle.	Doerr.
I.P.	0	1
I.C.	6	8
I.N.	3	1

The figures indicate the number of mice out of 30 in each instance dying within 10 days as a result of inoculation with either the Doyle or Doerr strains by the route shown.
I.P. = Intraperitoneal route. Dose, 0·2 c.c.
I.C. = Intracerebral route. Dose, 0·03 c.c.
I.N. = Intranasal route. Dose, 2 drops in nostril.

Susceptibility of White Rats to Infection with Fowl Pest.

In view of the different degrees of susceptibility exhibited by white mice to the variant forms of the fowl-pest virus, it was decided that a short experiment should be carried out to study the reaction of white rats to infection with these agents. For this purpose batches of 2 rats were inoculated with the Doyle and Doerr strains and with the canary and mouse variant of the Doyle strain by either the intraperitoneal, the intracerebral or the intranasal routes. The animals were kept under observation for a fortnight, and all deaths that occurred during this period are noted in Table IV. From this table it will be seen that the mouse variant is also highly pathogenic for white rats when given by any of the above routes. The canary variant only killed 1 rat out of 6, while neither the Doyle nor the Doerr strain produced any observable effect.

TABLE IV.—*The Comparative Pathogenicity in White Rats of Two Original and Two Variant Strains of the Fowl-pest Virus.*

	Strains of fowl pest.			
	Doyle.	Doerr.	Canary.	Mouse.
I.P.	0	0	0	1
I.C.	0	0	1	2
I.N.	0	0	0	2

Figures indicate the number of rats out of 2 dying in 14 days as a result of inoculation with one of the four strains of the fowl-pest virus by the route indicated.
I.P. = Intraperitoneal route. Dose, 0·5 c.c.
I.C. = Intracerebral route. Dose, 0·05 c.c.
I.N. = Intranasal route. Dose, 2 drops in nostril.

It has, of course, been known for some time that rats could be infected with fowl pest; thus Jansen and Neischulz (1934) showed that after intracerebral injection with virus, white rats died in 3–8 days with symptoms of paralysis, and Schmidt and Oerskov (1935) also successfully infected these animals with the Neischulz strain of fowl pest.

Immunity.

In order to determine whether the two variants formed from the original Doyle strain were antigenically similar to each other, to the parent strain and to another independent strain of fowl-pest virus, anti-sera were prepared

against the Doyle, Doerr, mouse and canary strains in the *rhesus* monkey—a species which, as will be shown in a subsequent communication, can be readily infected with fowl-pest virus. The monkeys after two or three injections of fowl-pest virus were bled at suitable intervals and the undiluted sera were mixed with equal amounts of dilutions of the mouse and canary strains. The mixtures, after standing overnight in the refrigerator, were injected intracerebrally into mice, 4 mice being inoculated with each dilution.

TABLE V.—*Immunity Experiments with Strains of Fowl-pest Virus.*

Serum.	Dilutions of virus.* Mouse strain.						Canary strain.					
	10^{-3}.	10^{-3}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.
Anti-mouse	4	4	1	0	0	0	4	3	2	0	0	0
Normal	4	4	4	3	1	0	4	4	4	2	1	0
Anti-canary	4	4	0	0	0	0	4	3	0	0	0	0
Normal	4	4	3	1	0	0	4	4	3	0	0	0
Anti-Doyle	4	3	0	0	0	0	4	4	3	0	0	0
Normal	4	4	3	1	0	0	4	4	4	4	2	0
Anti-Doerr	4	3	0	0	0	0	4	3	1	0	0	0
Normal	4	4	4	2	1	0	4	4	4	4	1	0

* Four mice inoculated intracerebrally with each dilution; the figure indicates the number of mice dying of fowl pest.

Batches of mice were similarly inoculated with the same dilutions of virus and normal monkey serum. The results show that all the sera prepared by the inoculation of any of the four strains had about the same virucidal action on the mouse and canary strains, killing from 100 to 1000 M.L.D. Unfortunately, the Doerr and Doyle strains were so slightly pathogenic for mice that no comparable figures could be obtained for the action of the immune sera on these two strains. Nevertheless the results obtained are sufficient to show that (1) no extraneous virus had been introduced as a result of intracerebral passage in canaries and mice, and (2) the Doyle virus and its two variants, if not identical, are at any rate closely related antigenically to the Doerr strain of fowl-pest virus.

These observations are further confirmed by the fact that mice which have survived intracerebral inoculation with the Doerr strain are solidly immunized against subsequent inoculation with the highly pathogenic mouse variant. Previous inoculation by the intranasal or intraperitoneal routes does not produce quite such a high degree of protection. Thus it would appear that in mice, immunization against fowl pest is dependent on the virus reaching the central nervous system.

DISCUSSION.

In this communication, which is only to be considered as an introduction to further experimental work, a description has been given of the method employed in the formation of two variant strains of the fowl-pest virus. The first of these, the canary strain, was produced by intracerebral inoculation in canaries. These birds, which are highly susceptible to the action of fowl-pest virus, were passively immunized before being inoculated, in order to

suppress, if possible, any generalized viscerotropic action that the virus of fowl pest may possess.

The second variant was produced by passaging the strain obtained from the canary intracerebrally in mice. The latter, it was found, could easily be infected with the canary variant, although they were only slightly susceptible to the action of the original or starting strain. The second or mouse variant differed from the canary variant and from the original strain in being even more highly pathogenic for mice, and, in fact, the varying degrees of pathogenicity in mice provided a ready means of distinguishing between these three " strains ".

The possibility that a contaminating virus might be responsible for certain of the results obtained has not been further considered, since it has been shown that the two variants and the original strain are antigenically related.

In a subsequent communication further details will be given regarding the activity of these fowl-pest variants, and of their pathogenicity in certain other animal species.

SUMMARY.

1. By intracerebral passage in passively immunized canaries, a variant of the fowl-pest virus has been produced.

2. Intracerebral passage of this canary variant in mice has led to the formation of a mouse variant.

3. These two variants and the original or starting strain can be differentiated from each other by their pathogenic effects in mice.

4. Both variants and the original strain are antigenically related.

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THE TRANSMISSION OF FOWL PEST TO FERRETS.

G. M. FINDLAY AND R. D. MACKENZIE.

Wellcome Bureau of Scientific Research, London.

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IN a previous communication (Mackenzie and Findlay, 1937) there was described the production of two variant strains of fowl-pest virus. These variants were derived from a parent strain that possessed a very feeble pathogenicity for mice by continued intracerebral passage (1) in canaries, and (2) in canaries and later in mice. In their effects on mice these two variants differed from each other and from their parent strain, although serologically they were still either identical or very closely related to one another. The three strains exhibited differences in their pathogenicity for rats.

Experiments are now described to show the varying infectivity of these strains for ferrets. In addition to the original strain obtained through the kindness of Dr. T. M. Doyle, of the Ministry of Agriculture Laboratory, Weybridge, and its two variants termed respectively the "canary strain" and the "mouse strain", comparison was made with a strain originally isolated in Switzerland by Prof. Doerr, of Zurich, and afterwards maintained in fowls by Dr. C. Todd and Mr. I. A. Galloway at the National Institute for Medical Research, Hampstead, to whom our thanks are due.

FOWL PEST IN FERRETS AND FITCHES.

Since Dunkin and Laidlaw (1926) first showed that the ferret is extremely susceptible to canine distemper it has been found to be readily infected with a number of viruses, and to all it reacts with the formation of broncho-pneumonic lesions. Thus Wilson Smith, Andrewes and Laidlaw (1933) found that the ferret was highly susceptible to influenza provided that the virus was instilled intranasally; later Francis and Magill (1935) showed that the strain of Rift Valley fever, maintained in the Rockefeller Institute, New York, also killed with broncho-pneumonia. The strain of Rift Valley fever virus maintained in this country for nearly six years fails, however, to kill ferrets, either by subcutaneous, intraperitoneal or intranasal inoculation, but produces only a short febrile reaction. The neurotropic strain of Rift Valley fever virus produced by Mackenzie and Findlay (1936), on the other hand, invariably kills ferrets on intracerebral and sometimes on subcutaneous inoculation, and, in addition to the lesions of encephalo-myelitis, broncho-pneumonia is invariably present in animals so killed.

The virus of fowl pest has been injected into ferrets, either intraperitoneally, intracerebrally, or by nasal instillation. Fitches (polecats) and ferrets, the albino variety of the polecat, have been used indiscriminately in these experiments, as the presence or absence of albinism appeared to make no difference to susceptibility. For the sake of convenience the term "ferret" is therefore

used to include both polecats and ferrets. The results obtained with the four strains are shown in Table I.

TABLE I.—*The Result of Inoculating Fowl-pest Virus into Ferrets by Different Routes.*

Strain.	Method of inoculation.					
	Intracerebral.		Intranasal.		Intraperitoneal.	
	Died.	Survived.	Died.	Survived.	Died.	Survived.
Mouse	5	0	3	0	4	0
Canary	3	0	3	0	0	2
Doyle	3	0	1	2	0	2
Doerr	0	3	0	3	0	2

Intraperitoneal inoculation.—Ten ferrets were inoculated intraperitoneally, 4 with the mouse strain and 2 each with the canary, Doyle and Doerr strains. All the ferrets inoculated with the mouse strain (1 c.c. of a 10 p.c. suspension of mouse brain) died 6 days after inoculation. There were no nervous symptoms beyond generalized twitching, but all 4 ferrets developed a febrile reaction within 24 hours of inoculation, the temperature varying between 105° and 108° F.; this reaction continued until shortly before death.

The 2 ferrets injected intraperitoneally with the canary strain (1 c.c. of a 10 p.c. suspension of canary brain in saline) developed fever on the 2nd and 3rd days after inoculation, but this fever only lasted for 3 to 4 days, after which the animals completely recovered. The 2 ferrets injected with the Doyle or parent strain (1 c.c. of a 10 p.c. suspension of hen liver) also exhibited a febrile reaction, but only 6 and 8 days after inoculation; here, again, the fever lasted for from 3 to 4 days (see Fig. 1). The 2 ferrets inoculated intraperitoneally with the Doerr strain (1 c.c. of a 10 p.c. suspension of hen liver) did not at any time show any significant rise of temperature (see Fig. 4).

There is thus a very definite gradation as a result of intraperitoneal inoculation of the four strains of fowl-pest virus, from an early rise of temperature and death, caused by the mouse strain, to a complete absence of symptoms after inoculation, with the Doerr strain.

Intranasal inoculation.—Twelve ferrets were first anæsthetized with ether, then 0·5 c.c. of the virus suspension was instilled into each nostril. The virus suspensions were similar in composition to those used for intraperitoneal injection.

Three ferrets were inoculated with the mouse strain and all died 6 days after inoculation. A febrile reaction, the temperature ranging between 105° F. and 108° F., began 24 to 48 hours after inoculation and continued till shortly before death, when there was a terminal fall (see Fig. 2). One ferret (No. 7) some 24 hours before death had considerable cough, its muzzle was stained with blood-red froth and its eyes were partially closed with exudate. In all 3 ferrets, for the last 24 hours of life there was weakness in the hind legs.

Three ferrets inoculated intranasally with the canary strain also died 6 days after inoculation. In 1 animal the temperature reached 105° F. 24 hours after inoculation, but in the 2 others the rise of temperature was delayed

till 48 hours after injection. Death was preceded by weakness in the hind legs. Three ferrets were inoculated with the Doyle strain ; 1 died on the 4th day after inoculation after a febrile reaction of only 48 hours, but without any nervous involvement ; the 2 others had a febrile reaction beginning on the 6th and 7th days after inoculation and lasting 2 and 3 days respectively. Both ferrets recovered.

Three ferrets inoculated intranasally with the Doerr strain showed a slight febrile reaction, and only in 2 of the 3 was there a rise to 104·8° and 105° F.

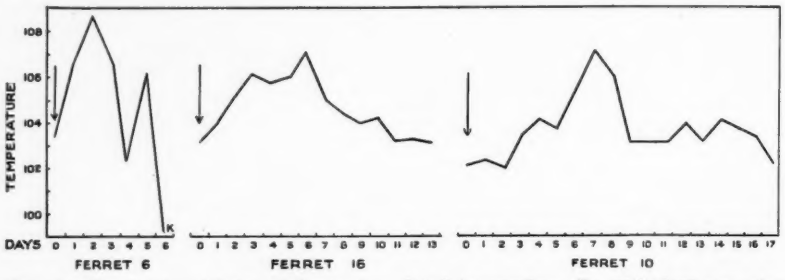

FIG. 1.—Temperature charts of ferrets inoculated intraperitoneally with fowl-pest virus. Ferret 6, mouse strain ; Ferret 16, canary strain ; Ferret 10, Doyle strain.

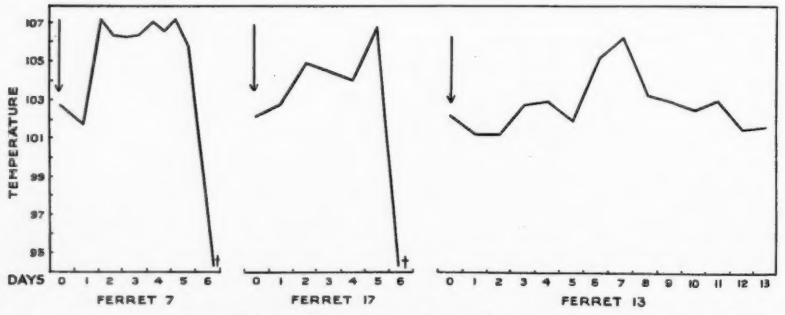

FIG. 2.—Temperature charts of ferrets inoculated intranasally with fowl-pest virus. Ferret 7, mouse strain ; Ferret 17, canary strain ; Ferret 13, Doyle strain.

in the 14 days following inoculation (see Fig. 4). By intranasal inoculation also a gradation in severity is shown by the four strains. The mouse and canary strains killed all the ferrets, the Doyle strain killed 1 of 3, while the Doerr strain failed to kill at all.

Intracerebral inoculation.—Fourteen ferrets were inoculated intracerebrally with fowl-pest virus, the inoculum consisting of 0·05 c.c. of a 10 p.c. suspension of mouse brain in the case of the mouse and canary strains, and 0·05 c.c. of a 10 p.c. suspension of hen liver in the case of the Doyle and Doerr strains. Three ferrets inoculated with the Doerr strain recovered, but all had febrile reactions beginning 24 hours after inoculation and lasting 6, 7 and 7 days

respectively (see Fig. 4). In these 3 ferrets, as the temperature fell a distinct weakness was noticeable in the hind legs, which, however, was not progressive, and was recovered from after 2 or 3 days. The other 11 ferrets inoculated with the Doyle, mouse and canary strains all died.

The 5 ferrets inoculated intracerebrally with the mouse strain developed a high temperature (106°–108° F.) (see Fig. 3) within 12 hours of inoculation, and 4 of them died within 36 hours, the other within 3 days of injection. Three ferrets inoculated intracerebrally with the canary strain also died in 5, 6 and

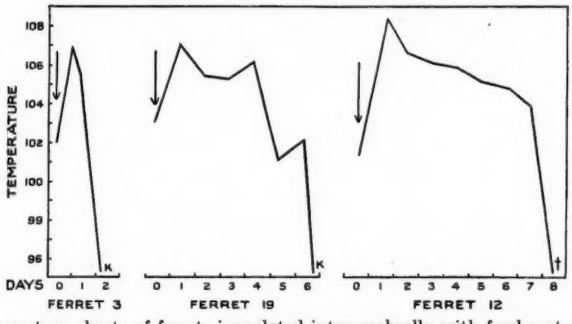

FIG. 3.—Temperature charts of ferrets inoculated intracerebrally with fowl-pest virus. Ferret 3, mouse strain; Ferret 19, canary strain; Ferret 12, Doyle strain.

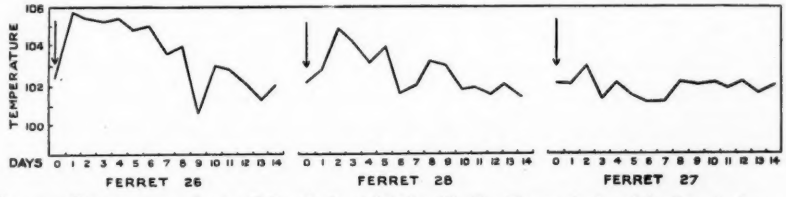

FIG. 4.—Temperature charts of ferrets inoculated with the Doerr strain of fowl-pest virus. Ferret 26, intracerebral inoculation; Ferret 28, intranasal instillation; Ferret 27, intraperitoneal inoculation.

6 days respectively. In these animals paresis of the hind legs, usually rather rather more pronounced on one side, appeared on the 2nd or 3rd day after injection and rapidly progressed upwards, involving the upper limbs and finally the neck; in some of the animals the head was forcibly retracted and there were tremors and convulsive movements.

Similar symptoms of what appeared to be an acute ascending myelitis were also seen in the 3 ferrets inoculated intracerebrally with the Doyle strain, though in these the time till death was slightly prolonged to 6, 7 and 8 days respectively. It will thus be seen that the strains of fowl-pest virus could be distinguished from one another, as in mice (Mackenzie and Findlay, 1937), by their relative pathogenicity when inoculated by different routes. The results obtained are summarized in Table I. Actual details of the ferrets inoculated are shown in Table II.

TABLE II.—*Inoculation of Ferrets with Fowl-pest Virus.*

No.	Strain.	Route of inoculation.	Result.
1	Mouse	i.c.	†36 hours.
2	,,	,,	†36 ,,
3	,,	,,	†36 ,,
4	,,	,,	†36 ,,
5	,,	,,	†3 days.
7	,,	i.n.	†6 ,,
22	,,	,,	†6 ,,
36	,,	,,	†6 ,,
6	,,	i.p.	†6 ,,
21	,,	,,	†6 ,,
34	,,	,,	†6 ,,
35	,,	,,	†6 ,,
18	Canary	i.c.	†6 ,,
19	,,	,,	†6 ,,
23	,,	,,	†6 ,,
17	,,	i.n.	†5 ,,
20	,,	,,	†6 ,,
25	,,	,,	†6 ,,
16	,,	i.p.	Recovered, fever 3–6 days.
24	,,	,,	Survived fever 1–2 days.
9	Doyle	i.c.	†6 days
12	,,	,,	†8 ,,
15	,,	,,	†7 ,,
11	,,	i.n.	†4 ,,
13	,,	,,	Recovered, fever 6–7 days.
14	,,	,,	,, ,, 7–9 ,,
8	,,	i.p.	,, ,, 8–9 ,,
10	,,	,,	,, ,, 6–8 ,,
26	Doerr	i.c.	,, ,, 1–8 ,,
29	,,	,,	,, ,, 1–7 ,,
30	,,	,,	,, ,, 1–8 ,,
28	,,	i.n.	,, ,, 2–3 ,,
32	,,	,,	Survived, no fever.
33	,,	,,	,, ,,
27	,,	i.p.	,, ,,
31	,,	,,	,, ,,

† = Death, followed by the number of days from inoculation to death.
i.c. = Intracerebral inoculation.
i.n. = Intranasal instillation.
i.p. = Intraperitoneal inoculation.

Although the symptoms noted during life pointed to a gradual involvement of the central nervous system, this was by no means the only system involved, as shown by the lesions found in the ferrets at autopsy. In the 22 ferrets that died there were present, in all but 2, well-marked broncho-pneumonic patches.

These broncho-pneumonic patches were unassociated with pleurisy or effusion into the pleural cavities, and as a rule were not confined to one lung, but were irregularly distributed in all the lobes of both lungs. In almost all ferrets the liver was paler than normal, with brownish-yellow patches, and in some it presented the typical box-wood tint which is characteristic of yellow-fever infections in man and rhesus monkey. There was no evidence of peritonitis, but the intestines were often slightly congested, and the mesenteric lymph-nodes were swollen and were the site of petechial hæmorrhages. The spleen was usually rather enlarged, and in some animals presented dark black irregular shaped areas, which stood out against the reddish-brown colour of the normal spleen. The kidneys varied in appearance ; in some animals they were relatively normal, in others they were pale yellowish-white in colour, the stellate vessels on the surface being very prominent. A trace of albumin was found in the urine in 2 ferrets, but in 4 others the urine was entirely free. The stomach was usually empty, but in almost all the ferrets there were larger or smaller amounts of blackish-brown pigment, the altered blood that is so characteristically seen in men, rhesus monkeys and hedgehogs dying of yellow fever. In 2 ferrets the blackish-brown pigment had passed down into the intestines. Blood-cultures from the heart and brain were invariably sterile, while smears made from broncho-pneumonic lung-tissue rarely revealed anything more than one or two stray micro-organisms. An account of the histological changes met with in ferrets will be given in a further communication.

DISTRIBUTION OF THE FOWL-PEST VIRUS IN THE TISSUES OF FERRETS.

The distribution of the virus in the tissues after intraperitoneal, intranasal and intracerebral inoculation of the mouse and canary strains of fowl-pest virus is seen in Table III. The ferrets were, as far as possible, bled out when nearly moribund, and a 10 p.c. suspension of the organs in saline was inoculated intracerebrally into batches of 6 mice each ; the number of mice dying is recorded. Ferret serum was found to be very toxic for mice when given by intracerebral injection, and the serum was therefore diluted 1 in 10 before inoculation.

TABLE III.

No.	Strain.	Route of inoculation.	Distribution of virus.*			
			Blood.	Brain.	Liver.	Spleen.
4	Mouse	i.c.	2	6	3	5
6	,,	i.p.	1	6	4	5
7	,,	i.n.	2	6	6	5
18	Canary	i.c.	2	5	3	5
20	,,	i.n.	1	6	6	5

* 6 mice inoculated in each batch ; the figures indicate the number dying.
i.c. = Intracerebral inoculation.
i.n. = Intranasal instillation.
i.p. = Intraperitoneal inoculation.

Virus was distributed in brain, liver and spleen, whatever the particular route of inoculation ; only a small amount was found in the blood.

IMMUNITY EXPERIMENTS IN FERRETS.

In a previous communication (Mackenzie and Findlay, 1937) evidence was brought forward to show that the Doerr, Doyle and the two derived mouse and canary strains were antigenically related from the fact that immune sera prepared against all four strains in rhesus monkeys were capable of neutralizing both the mouse and canary strains inoculated into mice.

As further evidence of immunity, ferrets which had recovered from the results of inoculation with the canary, Doyle and Doerr strains were, 3 weeks after their primary inoculation, reinoculated intraperitoneally with mouse-strain virus; the results are shown in Table IV. It will be seen that all the ferrets remained in perfect health without any rise of temperature which could be attributed to the inoculation of the mouse-strain virus, though this virus was always fatal to normal ferrets on intraperitoneal inoculation.

TABLE IV.

No.	Strain of primary inoculation.	Route of primary inoculation.	Result of subsequent intra-peritoneal inoculation with mouse strain.
8	Doyle	i.p.	No fever.
10	,,	,,	,,
13	,,	i.n.	,,
14	,,	,,	,,
16	Canary	i.p.	,,
24	,,	,,	,,
26	Doerr	i.c.	,,
29	,,	,,	,,
27	,,	i.p.	,,
28	,,	i.n.	,,

i.c. = Intracerebral inoculation.
i.n. = Intranasal instillation.
i.p. = Intraperitoneal inoculation.

THE RELATIONSHIP BETWEEN THE FOWL-PEST AND OTHER VIRUSES PATHOGENIC FOR FERRETS.

In view of the presence of broncho-pneumonia in ferrets inoculated with fowl-pest virus, it seemed to be of interest to determine whether any generic relationship existed between fowl-pest and the viruses of canine distemper, influenza and Rift Valley fever, all of which are capable of inducing a fatal pneumonia in the ferret. Through the kindness of Dr. C. H. Andrewes, of the National Institute for Medical Research, to whom our thanks are due, we received a supply of horse-serum which contained immune bodies both to human and swine influenza viruses. Dr. R. A. O'Brien, of the Wellcome Physiological Research Laboratories, Beckenham, was good enough to supply us with anti-distemper serum, while a supply of anti-Rift Valley fever serum was available in these laboratories.

Mixtures of each of these three antisera and increasing dilutions of the mouse strain of fowl-pest virus were made and inoculated intracerebrally into

mice ; as controls a fowl-pest antiserum made in the rhesus monkey against the mouse strain of fowl pest, and a normal monkey serum were also mixed with dilutions of virus and similarly inoculated into mice.

The results are shown in Table V.

TABLE V.—*Serum.**

Dilution of virus.	Anti-fowl pest.	Anti-distemper.	Anti-influenza.	Anti-Rift valley fever.	Normal monkey.
10^{-1}	4	4	4	4	4
10^{-2}	2	4	4	4	4
10^{-3}	1	4	4	4	4
10^{-4}	0	2	4	4	4
10^{-5}	0	2	3	4	4
10^{-6}	0	0	0	2	1

* 4 mice inoculated intracerebrally with each dilution ; the figure indicates the number dying.

No evidence was obtained of any relationship between the virus of fowl pest and either influenza, distemper or Rift Valley fever viruses. In order, however, to make doubly sure that there was no relationship to influenza, Dr. Andrewes very kindly tested anti-fowl-pest serum from the rhesus monkey against influenza virus in mice, and again no evidence was obtained that the anti-fowl-pest serum contained any immune bodies against influenza virus. In addition certain clinical and pathological findings distinguish the two infections in the ferret. Thus, in fowl pest, the virus is not restricted to the nasal passages, the temperature, as shown by Wilson Smith, Andrewes and Laidlaw (1933), is not typically diphasic and catarrhal symptoms are not common.

One other point of differentiation between fowl-pest and influenza virus lies in the reaction of the pig to fowl pest. Intranasal instillation of 2 c.c. of fowl-pest virus into a pig produced no rise of temperature and no symptoms of any sort. Intracerebral injection of 0·5 c.c. of a 10 p.c. suspension of mouse brain infected with fowl-pest virus caused a rise of temperature from 104° to 106·6° F. 48 hours after inoculation. The temperature remained up for 24 hours and then fell to 103·8° F., remaining normal thereafter. Both after intranasal and intracerebral inoculation immune bodies developed in the serum of the pigs against the virus of fowl pest.

Preliminary observations only have so far been made on one interesting point in which there does seem to be a similarity between the behaviour of fowl pest and influenza in the ferret. It will be remembered that Andrewes, Laidlaw and Smith (1934) found that influenza virus was not directly transmissible from man to mouse unless it had been passaged through the ferret.

In the case of Ferret 9, which died after an intracerebral inoculation of the Doerr strain virus, it was found that the virus present in the brain was highly pathogenic for mice, since on intracerebral inoculation with a suspension of the ferret brain, 5 out of 6 mice died in 2, 2, 2, 4 and 4 days, while intracerebral inoculation of mice with the Doerr strain of virus rarely kills mice in under 7 days, and then in only a small percentage of cases. This question is being further investigated.

DISCUSSION.

As in a previous communication (Mackenzie and Findlay, 1937) it was shown that by their pathogenicity for mice the strains of fowl pest could be differentiated, so now it has been possible with ferrets to differentiate the same four strains from each other. The Doerr strain was the least pathogenic for ferrets, since it only produced a fatal febrile illness, associated with weakness in the hind legs, on intracerebral inoculation ; no particular reaction followed intranasal or intraperitoneal inoculation. The Doyle strain, on the other hand, killed on intracerebral inoculation ; 1 out of 2 died after intranasal instillation ; 2 out of 2 survived intraperitoneal inoculation. All the ferrets exhibited a very well-marked febrile reaction. The canary strain killed by intracerebral inoculation and intranasal instillation, but not by intraperitoneal injection. The mouse strain killed when given by any route.

Whether this difference in pathogenicity for ferrets and mice would remain after repeated passage through the ferret is a question that remains for further investigation. A point of particular interest is the fact that though the predominant clinical symptoms were referable to an involvement of the central nervous system, the lungs, liver and other viscera were also the site of definite lesions. The presence of severe broncho-pneumonia following not only intranasal instillation, but intracerebral and intraperitoneal inoculation, once again emphasizes the peculiar reaction of the ferret to a number of viruses.

Fowl pest is, however, the first virus to be found capable of producing lesions in the abdominal viscera of ferrets which are similar to those produced in man and susceptible animals by the viruses of yellow fever and Rift Valley fever. The presence of these lesions suggests in fact that the virus of fowl pest must fall into the same group of pantropic viruses as yellow fever, Rift Valley fever, dengue, equine encephalomyelitis and possibly horse-sickness.

CONCLUSIONS.

1. The ferret and fitch are highly susceptible to the virus of fowl pest.
2. One strain of fowl pest killed ferrets on intracerebral and intraperitoneal inoculation as well as on intranasal instillation ; a second failed to kill on intraperitoneal inoculation, but killed after intracerebral inoculation and intranasal instillation ; another strain failed to kill, but produced a febrile reaction on intracerebral inoculation.
3. The pathogenicity of fowl-pest strains for ferrets paralleled their pathogenicity for mice.
4. All four strains produced cross-immunity in ferrets.
5. The clinical symptoms produced in ferrets by the fowl-pest virus were those of an ascending myelitis.
6. At autopsy there were also found broncho-pneumonia, and lesions of the liver, kidney and stomach.
7. No relationship could be found between the virus of fowl pest and other viruses—canine distemper, influenza and Rift Valley fever—which induce broncho-pneumonia in ferrets.
8. The pig inoculated intranasally with fowl-pest virus exhibits no reaction,

but develops immune bodies; inoculated intracerebrally it develops a short febrile reaction.

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CENTRIFUGATION STUDIES: III. THE VIRUSES OF FOOT-AND-MOUTH DISEASE AND VESICULAR STOMATITIS.*

W. J. ELFORD AND I. A. GALLOWAY.

From the National Institute for Medical Research, London, N.W. 3.

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EARLIER ultrafiltration studies on the viruses of foot-and-mouth disease and of vesicular stomatitis showed that these viruses differ considerably in particle size (Galloway and Elford, 1933). Thus the virus of foot-and-mouth disease was found to be 8–12 mμ., in diameter, and that of vesicular stomatitis was about eight times as large, 70–100 mμ. Recently one of us (Elford, 1936) has described a new technique whereby the particle sizes of viruses may be determined from their rates of sedimentation in centrifugal fields, and we report here the results obtained in studies with the two viruses mentioned above.

The details of the experimental method have been given fully in earlier papers (Elford, 1936; Elford and Andrewes, 1936), and will not be elaborated here. It suffices to say that the suspension is spun while contained within an inverted capillary (0·2 cm. or less in diameter), which during the spinning dips, to an extent corresponding to the length of the capillary, into the suspension contained in the centrifuge bucket. At the end of the run for time, t, the capillary cell is withdrawn and its contents tested to determine the average concentration, C_t, within the capillary, and this is compared with the concentration, C_o, of the unspun control. Then, knowing the speed of the centrifuge, N, in r.p.m., the density, σ_p, of the sedimenting particles, and the values, σ_m, and η, the density and viscosity of the medium respectively, one may calculate the particle diameter, d, from the relationship—

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{\eta \log. \dfrac{x_1 + l}{x_1 + l\, C_t/C_o}}{(\sigma_p - \sigma_m)\, N^2\, t}} \quad . \quad . \quad . \quad (1).$$

* This work was done on behalf of the Foot-and-Mouth Disease Research Committee, who have given their permission for the publication of this paper.

d is given in mμ. when t is expressed in mins., x_1 the distance in cm. from the axis of rotation to the top of the capillary, and l the length of the capillary in cm.

FOOT-AND-MOUTH DISEASE VIRUS.

Application of the inverted capillary tube technique in studies with the smallest bacteriophage, S 13, had shown that, for capillaries 1 cm. in length, centrifugation at 11,000 r.p.m. (effective centrifugal force = 14,000 g.) for a period of 3 hours resulted in the average concentration of phage within the capillary being reduced to about 50 p.c. of the original (Elford, 1936). Since S 13 phage and the virus of foot-and-mouth disease had been found to have the same particle size by ultrafiltration analysis, it was anticipated that under the above conditions of centrifugation no significant change in the concentration of foot-and-mouth disease virus would be detected. (A significant change in the concentration of a virus when tested by inoculation of a susceptible animal with serial dilutions of the liquid is generally not less than a tenfold difference.) This prediction was confirmed in a series of six experiments with the G.F. strain of the virus (Vallée O type), using highly potent filtrates of diluted vesicle lymph and also of purified virus (see Galloway and Elford, 1936) in a broth and $M/30$ phosphate medium. The results indicated that 3 hours' spinning at 11,000 r.p.m. in capillaries 1 cm. in length might produce a slight drop in the average concentration of virus within the capillaries, possibly to one-fifth of the original (tests were made on serial fivefold dilutions). In view of this, an attempt was made to render the method more sensitive, so that with the same centrifugal force a greater change in the average concentration within the capillary might be produced. To achieve this, capillaries only 0·5 cm. long and 0·1 cm. wide were employed. The difficulty presented by the very small volume of fluid contained in such capillaries was surmounted by having, in each inverted metal cell, nineteen of these capillaries drilled in a parallel arrangement similar to that illustrated in the first paper of this series (Elford, 1936). Then, when the contents of the capillaries in a pair of such metal cells were pooled, about 0·15 c.c. of liquid was available for test. Under these new conditions the value of x_1 in equation (1) is 7·75, and $l = 0{\cdot}5$. Hence the relationship for calculating the particle diameter is—

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{\eta \log. \dfrac{8{\cdot}25}{7{\cdot}75 + 0{\cdot}5\, C_t/C_o}}{(\sigma_p - \sigma_m)\, N^2\, t}} \quad . \quad . \quad . \quad (2).$$

The experimental results obtained with the short capillaries are given in Table I. The magnitude of the change in the concentration of virus as the result of spinning was now one which was very readily measured. It should be mentioned here that very careful tests were made to determine whether or not the metal (M.V.C. alloy) of which the cells were made exerted any harmful effect on the virus. No such effect could be detected. The medium used in all centrifugation experiments has been Hartley's broth diluted with an equal volume of $M/30$ phosphate buffer at pH 7·6.

In some of the experiments S 13 phage was added to the filtrate of virus to provide additional comparative data. Generally the drop in concentration of

the virus appeared to be somewhat higher than of the phage, but when allowance is made for the relative accuracies of the methods for estimating concentrations in the two instances, the rates of sedimentation may be regarded as very similar.

Tests were made to see whether or not the virus could be resuspended from the filter-paper which was inserted at the bottom of the bucket to prevent redispersion of sedimented particles. The filter-paper was repeatedly squeezed and saturated in the liquid contained in the bucket after the capillary had been withdrawn. The virus could be recovered completely. Parallel tests in the case of S 13 phage showed in every instance that the titre of the resuspended phage was greater than that of the unspun control, indicating an " activation " to the extent of about fivefold. This " activation " phenomenon has been more fully investigated with interesting results, which will be reported separately later.

It will be seen from the table that the virus particles contained in the ultrafiltrate through a 40 mμ. membrane showed the same rate of sedimentation as virus contained in the stock filtrate through a 0·5 μ membrane. Also an experiment with the virus contained in the residue above a 58 mμ. membrane showed that even here the particles sedimented no more readily. This supported the evidence of a subsequent filtration experiment in which the virus in the 58 mμ. residue passed through a 40 mμ. membrane. The original retention of virus above the 58 mμ. membrane would appear to have been due to a statistical sieving effect, and not because the particles were larger than the average.

The data of Table I indicate that, by the use of the 0·5 cm. inverted capillary cells, the concentration of foot-and-mouth disease virus is reduced to one-tenth the original value (*i. e.* $C_t/C_o = 0{\cdot}1$) by spinning for 2 hours at 11,000 r.p.m. Hence using the relationship expressed in equation (2)—

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{\eta \log. \frac{8{\cdot}25}{7{\cdot}75 + 0{\cdot}05}}{(\sigma_p - \sigma_m)\,(11{,}000)^2\,120.}}$$

Direct measurement gave the values $\eta = 0{\cdot}011$ and $\sigma_m = 1{\cdot}009$ for the medium at 25° C.

In order that d may be calculated it is also necessary to know the value of σ_p, the density of the virus particles. This is difficult to determine reliably, but evidence (to be reported later) has been acquired by spinning the virus in media of different known densities that σ_p has a probable value very near to 1·30. When this value is used the particle diameter of the virus is calculated to be 20 mμ.

VESICULAR STOMATITIS VIRUS.

The virus of vesicular stomatitis (" Indiana " strain used) was found to sediment with relative ease compared with the virus of foot-and-mouth disease. Thus when the former was suspended in a mixture of equal parts of broth and saline at pH 7·4, centrifugation in the 1 cm. inverted-capillary cells for 3 hours at 10,000 r.p.m. reduced the concentration to between one-hundredth and one-thousandth the original. A series of experiments at the

TABLE I.—*Sedimentation Data for the Virus of Foot-and-Mouth Disease.*

Experiment.	Speed (r.p.m.).	Time (mins.).	C_o.	C_t.	C_t/C_o.	Remarks.
1	11,800	180	10^{-4} ++	10^{-1} ++	0·001	0·5μ membrane filtrate. C_t/C_o for S 13 phage control = 0·046.
2	10,750	120	10^{-3} ++	10^{-2} ++	0·1	0·5μ membrane filtrate. C_t/C_o for S 13 phage control = 0·25.
3	10,750	120	10^{-4} ++	10^{-3} +−	0·1–0·01	0·5μ membrane filtrate.
4	10,850	120	10^{-2} +−	10^{-1} +−	0·1	40 mμ. „ „
5	10,850	120	10^{-3} +−	10^{-2} +−	0·1	40 mμ. „ „
6	11,000	120	10^{-2} +−	10^{-1} ++	0–0·1	Fraction retained above a 58 mμ. membrane.
7	11,000	180	10^{-5} +−	10^{-3} +−	0·01	0·5 μ membrane filtrate.

Length of capillary = 0·5 cm.

The medium was in each case Hartley's broth diluted with an equal volume of $M/30$ phosphate buffer at pH 7·4. Density of medium = 1·009; viscosity = 0·011.

C_o and Ct are given as the limiting infective dilution; tests were made in duplicate on serial tenfold dilutions in all cases.

++ = Both guinea-pigs inoculated showed lesions. +− = One of two guinea-pigs inoculated showed lesions.

TABLE II.—*Sedimentation Data for the Virus of Vesicular Stomatitis.*

Experiment.	Medium.	Speed (r.p.m.).	Time (mins.).	C_t/C_o guinea-pig test.	C_t/C_o egg membrane test.	Remarks.
1	Saline	10,000	180	0·01–0·001	..	In all experiments except the 5th membrane filtrates of suspensions of vesicle fluid were used.
	Broth	„	„	0·01–0·001	..	
2	„	„	„	0·01	1/125 to 1/625	
	„	„	30	0·1	1/5 to 1/25	In Experiment 5 a washed preparation of the virus was used.
3	Saline + broth 1 : 1	„	60	Less than 0·1	1/25	
4	„ „	„	33	..	1/25	Egg membrane tests made on serial five-fold dilutions.
5	„ „	„	30	..	1/5 to 1/25	
6	Saline + broth	9800	8	0–0·1	..	In Experiments 1–5 length of capillary (l) = 1 cm. In Experiments 6 and 7, l = 0·5 cm.
7	„ „	9600	15	0·1	..	

Density of the saline + broth medium at 25° C. = 1·009.
Viscosity „ „ „ 25° C. = 0·010.

same speed of 10,000 r.p.m. but with different durations of spinning enabled the conditions required to produce a tenfold drop in virus concentration to be determined (see *Table II*). Membrane filtrates of broth + saline suspensions of vesicular fluid from the pads of infected guinea-pigs were first studied, and it was found that spinning for 30 minutes at 10,000 r.p.m. gave $C_t/C_o = 0{\cdot}1$. Virus which had been washed free of demonstrable protein on a suitable membrane behaved in the same way. The virus was detected by two methods, (*a*) by intradermal inoculation in the pads of guinea-pigs, and (*b*) by allowing the virus to propagate in the chorio-allantoic membrane of the developing hen's egg (see Burnet and Galloway, 1934). While the latter provided the more sensitive test for the presence of the virus, the proportional changes in concentration indicated by the two methods were in complete accord.

In calculating the particle size of the virus of vesicular stomatitis the density of the virus will be taken as 1·20. [*Cf.* The values found for the densities of vaccinia virus, 1·18, and influenza virus, 1·20, in sugar solutions (Elford and Andrewes, 1936).]

Thus from relationship (1) adapted for $l = 1$ and $x_1 = 7{\cdot}25$ and with the conditions for $C_t/C_o = 0{\cdot}1$ cited above—

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{0{\cdot}010 \log. \dfrac{8{\cdot}25}{7{\cdot}25 + 0{\cdot}10}}{(1{\cdot}20 - 1{\cdot}009)\ (10{,}000)^2\ 30}} = 74,$$

i. e. the particle diameter of vesicular stomatitis virus is 74 mμ. If the density of the virus proved to be as high as 1·30, then the calculated particle diameter would be 60 mμ.

Two experiments, Nos. 6 and 7, *Table II*, were made with the cells having short capillaries 0·5 cm. in length. The concentration of the virus of vesicular stomatitis was reduced tenfold by spinning for 15 minutes at about 10,000 r.p.m. Under similar conditions of centrifugation it was necessary to spin the virus of foot-and-mouth disease for 2 hours at about 11,000 r.p.m. to effect the same degree of sedimentation. This, too, would indicate that, if the particles have the same densities, the virus of vesicular stomatitis is about three times as large as the virus of foot-and-mouth disease.

DISCUSSION.

The measurements of the rate of sedimentation of the virus of foot-and-mouth disease described here are, to our knowledge, the first to be recorded for this virus. Busch (1934), adopting the procedure of Bechhold and Schlesinger (1931), was unable to detect any sedimentation after spinning a suspension of the virus in phosphate buffer solution for 4 hours at 10,000 r.p.m., and he concluded that the particle size of the virus must be less than 30 mμ. We too, in the past, have failed to obtain any evidence that the virus could be thrown down when using wide centrifuge tubes. However, it is now clear that when conditions obtain, such as are provided by the inverted-capillary tube technique, where the centrifugal motion of the particles is undisturbed by " mixing ", then the sedimentation of even the smallest of the known viruses can be measured.

The value 60–74 mμ. found by centrifugation for the particle diameter of the virus of vesicular stomatitis is in fair agreement with our earlier estimation by ultrafiltration—70–100 mμ. On the other hand, the particle diameter of the virus of foot-and-mouth disease calculated from centrifugation data is about twice the average given by ultrafiltration, viz. 20 mμ. as compared with 8–12 mμ. Our recent centrifugation experiments with S 13 bacteriophage (also 8–12 mμ. by ultrafiltration) suggest that it, too, is close to 20 mμ. in size. In seeking an explanation of this difference between the results of centrifugation and ultrafiltration, the following points are to be considered: (1) The figure obtained by ultrafiltration may be somewhat low owing to the factor used in translating the end-point membrane porosity in terms of particle diameter being too small. Experience with the proteins (Elford, 1933; Elford and Ferry, 1934), and consideration of such factors as the relative sensitivities of the tests for proteins and viruses, and the nature of the medium containing the suspended particles, lead to the view that the ultrafiltration figure may be somewhat too low.

(2) The chief uncertainty attaching to the centrifuge method concerns the true density of the virus particles, the determination of which is rendered very difficult owing to the small amounts of material available, and the lack of precision in the methods of estimating concentration. Studies of the sedimentation in media of different known densities can give helpful information, but in the absence of precise knowledge of any dehydrating influence that may be exerted by high solute concentrations on viruses and phages, the possibility of serious error must not be overlooked. On this account some revision of the particle sizes calculated from centrifugation data may be found necessary as more accurate density values become available.

It may be of interest to note that should the density of the virus prove to be greater than 1·30, say 1·40, then the calculated particle diameter would be 17 mμ. instead of 20 mμ.

(3) The particles being studied may not be spherical in shape, but elongated. Ultrafiltration would give evidence of the smallest dimension. Likewise when there is any departure from homo-dispersity the information on particle size refers to the smallest phase present, since by the ultrafiltration method the limiting membrane porosity required to retain completely all the virus is determined. However, the forms of the filtration end-point curves for foot-and-mouth disease virus and also for the phages have been found to parallel closely those given by known monodisperse systems like the proteins, of which the particles are spherical or very nearly so.

The question of the degree of uniformity in size of virus particles is a very important one, and we have investigated it further in the case of foot-and-mouth disease virus. Analysis by ultrafiltration indicates a uniformity in the size of the particles, the virus appearing without loss in filtrates through membranes of progressively lower porosities until the "end-point" is approached (Galloway and Elford, 1931). In the present study measurements have been made of the sedimentation of (*a*) the virus particles contained in a 0·5μ membrane filtrate, (*b*) those particles passing a 40 mμ. membrane, and (*c*) those virus particles contained in the residue above a 58 mμ. membrane after over 90 p.c. had filtered through. In all cases the virus showed the same

rate of sedimentation. No evidence suggesting pleomorphism of the virus can be deduced from the centrifugation or the ultrafiltration experiments. The virus particles appear to be of relatively uniform size, approaching 20 mμ. in diameter, the probable outside limits being 10–20 mμ.

Recently Levaditi, Païc, Krassnoff and Voet (1936) have given a value 3–5 mμ. for the size of the virus particles of a Vallée A strain of the foot-and-mouth disease virus. This interested us very much, since all the strains of foot-and-mouth disease virus which we have examined have shown the same filtration end-point—25 mμ. (Galloway and Elford, 1931). Prof. Levaditi very kindly sent us some of the Vallée A strain of foot-and-mouth disease virus which he had employed, and we find that both in ultrafiltration and in centrifugation experiments (in collaboration with Dr. Schlesinger, to be reported later), this strain behaves exactly as the other strains investigated. All have the same particle size.

SUMMARY.

1. The inverted-capillary tube technique for studying the behaviour of suspensions in centrifugal fields has been successfully applied in studies with the virus of foot-and-mouth disease—one of the smallest of known animal viruses. Measurements of the rate of sedimentation of the virus are recorded, and its particle size for a density value 1·30 is calculated to be 20 mμ. This result is discussed in relation to the earlier value given by ultrafiltration analysis.

2. Evidence from centrifugation experiments is advanced, supporting that previously given by ultrafiltration analysis, for the view that the particles of the virus of foot-and-mouth disease are relatively uniform in size. All strains, too, are found to have the same particle size.

3. Measurements of the rate of sedimentation of the virus of vesicular stomatitis are also recorded. The particle diameter of this virus is found to be 74 mμ. or 60 mμ., according as to whether the density of the particles is assumed to be 1·20 or 1·30 respectively.

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THE MEASUREMENT OF THE SIZE OF VIRUSES BY HIGH-SPEED CENTRIFUGALIZATION.

JAMES McINTOSH AND F. R. SELBIE.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, W. 1.

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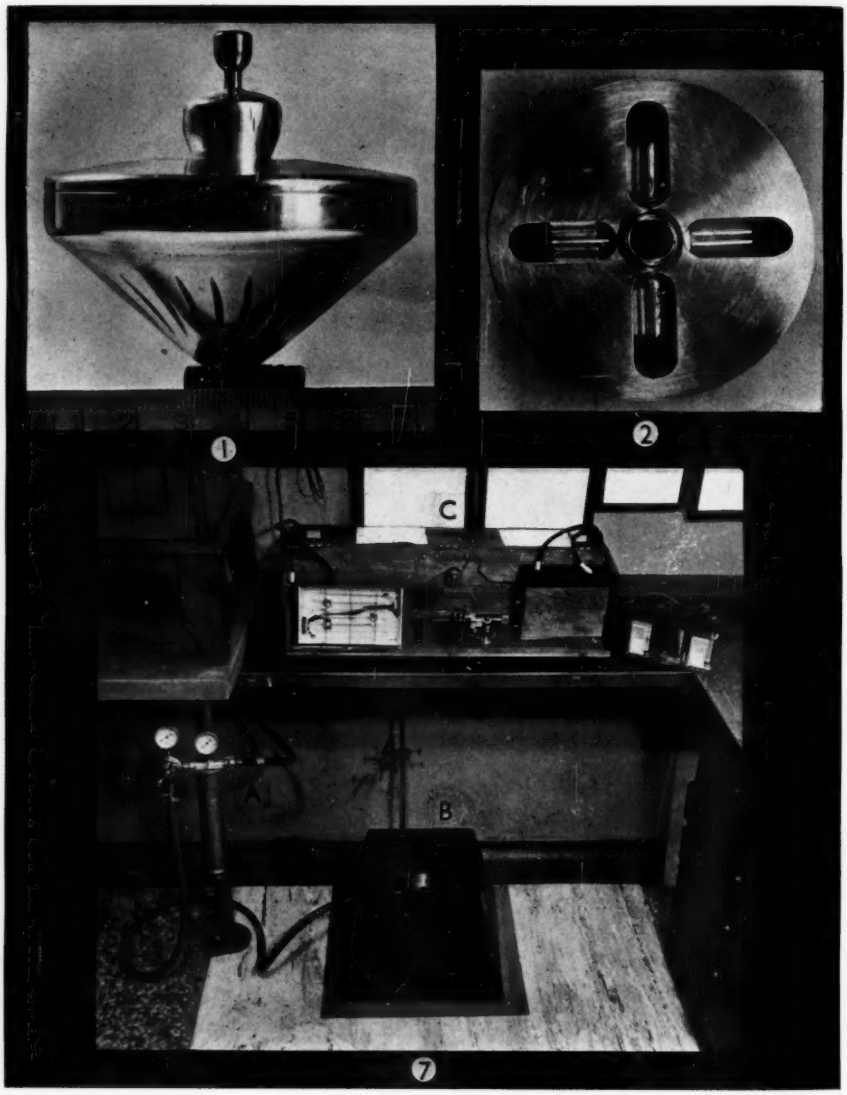
MEASUREMENT of size is an important factor in the identification of viruses, and in recent years numerous investigations have been made on this subject. At the present there is a choice of three procedures for such measurements: (1) Microscopical observation, (2) filtration, and (3) centrifugalization. The minute size of many viruses precludes direct microscopical observation except in the larger forms, and the size of most of them has been determined by their passage through filter candles or membranes of different porosities; from these much useful information has been obtained. Criticism, however, has been directed against the accuracy of this procedure owing to the difficulty of estimating the size of the pores in the filters, and also the actual relationship between the size of the filter pores and that of the viruses. For this reason Bechhold and Schlesinger (1931), Elford and others (1936) have recently made use of centrifugalization to determine the size of the particles.

Some time ago an attempt was made in this Institute to test the particulate nature of the Rous sarcoma virus by centrifugalization by means of an ordinary laboratory high-speed centrifuge, but the results were unsatisfactory; apparently vibration and heating by the production of convection currents prevented sedimentation. About this time our attention was directed toward the possible use of the high-speed rotors made on the principle of Henriot and Huguenard (1925, 1927), and used as centrifuges by Beams, Weed and Pickles, McBain, Gratia and others in America and on the Continent. A centrifuge was devised on this principle, and was found to be capable of speeds up to and over 60,000 revolutions per minute. It was shown (McIntosh, 1935) that this centrifuge could sediment completely the Rous sarcoma virus after spinning for half to one hour, and even produce a 100-fold deposition of *B. coli* bacteriophage.

The Centrifuge.

Numerous observations have now been made on the sedimentation of bacteria and viruses, and so constant and reliable have the results been that it is thought best to give full particulars of the centrifuge along with our experimental findings. As we have already indicated, our centrifuge is based on the principle devised by Henriot and Huguenard, used by Beams and others in America. Henriot and Huguenard made use of the suction power and driving force produced when a jet of air of high pressure is allowed to escape from small orifices. This machine has no bearings and simply spins on a cushion of air—the suspending and driving force is supplied by compressed air escaping



McIntosh and Selbie.

UNIV. OF MICH.

through a ring of small holes which impinges on a series of cuts on the under-surface of the rotor. The centrifuge consists of three essential parts: (*a*) the rotor, (*b*) the stator, and (*c*) the compressor.

The rotor (Fig. 1).—This consists of an inverted cone of duralumin measuring 7·6 cm. in diameter at the base, with a vertex angle of 110°. The top of the base is flat, with four radial recesses cut in it, capable of taking four small test-tubes measuring 2·0 cm. by 0·5 cm. (Fig. 2). The test-tubes are held in place during the spinning by a flat disc of duralumin screwed down on to the cone. These test-tubes are of such a diameter that they will not allow broth or similar fluid to run out if placed on their sides. The central duralumin screw (left-hand thread) for holding the cover on has a hard steel pin with a

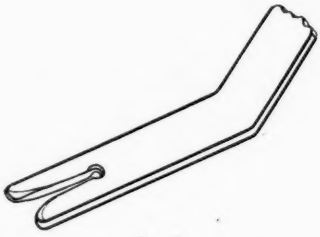


FIG. 3.

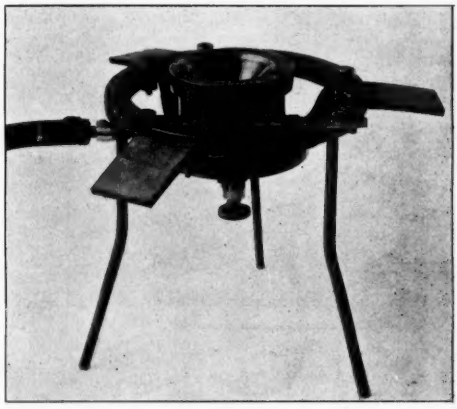


FIG. 4.

head driven through the screw and pinned, care being taken that the pin is as near as possible to the true centre of the rotor. This hardened pin and knob is devised for the purpose of lifting the rotor off the stator with the aid of a brass fork or prong (Fig. 3) when the spinning has been completed. The driving force of the escaping air is assisted by the series of cuts or grooves arranged either in a radial or helicoidal manner on the under-surface of the rotor.

The stator (Fig. 4) is a hollow cone made of an easily worked metal, such as phosphor-bronze, with a series of nine holes round the side of the cone to allow the compressed air to emerge, in an upward and anti-clockwise manner. The internal diameter of the stator at the top is 6·0 cm. The angle of the stator according to Henriot and Hugeunard should be about 90°; we have found that angles of 90° or 92° have been equally satisfactory. In the construction of the stator one of the most important parts is the drilling of the small holes or jets. They are drilled about 2·0 cm. down from the upper and inner margin of the cone, and at an angle of 45° (angle *a*, Fig. 5) to the axis of the cone when viewed antero-posteriorly and vertically when viewed from the side.

By this means the air is directed upwards and to one side, so as to give a driving vortex of compressed air. For our purpose the most suitable size of drill has been a No. 63. It is essential for the smooth running of the rotor that these small holes should be drilled in an exact and uniform manner. There are considerable technical difficulties in drilling these correctly on the slant, and care has to be taken to overcome this. One method is, before machining, to drill holes at the correct angle from the outside of the stator and then to tap in a screw jet, as is shown in the working drawings (Fig. 5).* Beams suggested

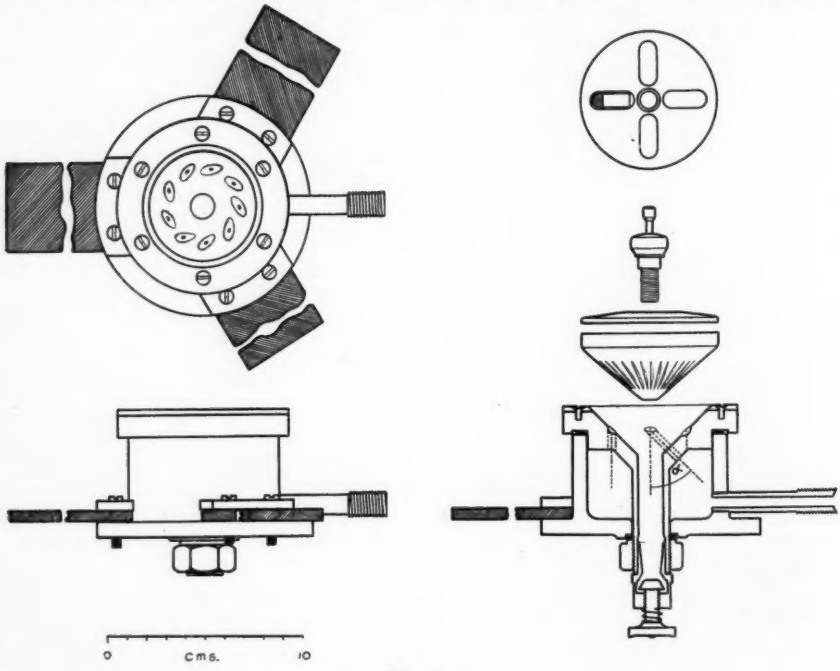


FIG. 5.

that the upper edge of the stator should be covered with a hard metal such as a steel ring to prevent any wear, and that was done with our large stator, but another has proved quite satisfactory without the hard edge. Beams also suggested having a hole at the bottom of the stator as it permitted steadier running. We found, however, that this is true only at certain speeds, as the size of the hole required varies with the speed ; this difficulty was overcome by fitting an adjustable valve. By closing the valve slightly after the rotor has been spinning for some time higher speeds are obtained—in fact the rotor can be run with the valve almost closed, but it cannot be started with the valve in this position owing to the increased suction pulling the rotor down on

* We are indebted to Messrs. Baird and Tatlock, London, for the accurate manner in which they carried out this work.

the stator. This is known to occur if a rattling noise is heard; if the valve is then opened slightly the rotor will spin freely again.

Finally, to prevent jarring, the stator should be suspended by rubber from a suitable stand and, whilst spinning, the centrifuge should be covered by some kind of protective box or case.

The compression plant.—Owing to the size of the rotor and the total area of the emission jets a large capacity air-compressor is essential if high speeds (60,000 and over) are to be maintained. We have used a machine capable of giving 20 c. ft. per minute at a pressure of 80–90 lb. per sq. inch, such as is made by the B.E.N. Patents Ltd., London. This actual compressor is driven by a 4 h.p. electric motor. A smaller compressor will work this centrifuge quite well when the higher speeds are not required, as the machine will run at 8–10 lb. pressure. With the larger compressor plants good filtration of the air after compression is necessary to remove particles of oil and dirt, which otherwise would be carried up the tubing and block the small holes of the stator. Pressure gauges and some form of variable air-pressure valve are essential.

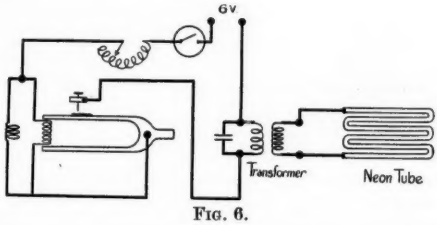


FIG. 6.

The revolution counter.—In order to get constant conditions of spinning we found that it is essential to have some form of revolution counter. For this purpose we used a stroboscope (Fig. 6), consisting of a neon light worked at a known frequency (100 per second) by an electrically driven tuning-fork. This gives a frequency of 6000 per minute. To use this stroboscope a V-shaped blue or black mark is painted on the upper surface of the rotor, with the apex of the V directed to the central axis. The neon lamp is held in a clamp just above the rotor; the pressure is turned on, say, to 30–40 lb., the rotor held lightly between the thumb and forefinger of both hands and held in position. The rotor is immediately gripped in the vortex of air and spins rapidly at increasing speed. When a speed of 6000 revolutions is accomplished the V mark appears single and stationary. As the speed increases, at each multiple of the 6000 revolutions the V mark again becomes stationary; thus, with a pressure of 60 lb. the top speed is reached only after the V has become stationary six times—giving a speed of $6 \times 6000 = 36{,}000$. By this means not only can direct multiples be counted, but also halves and quarters, as at half a multiple there are two V's visible and at quarters, four. With the aid of good variable air-pressure valves the speed selected can be maintained accurately for hours at a time, and all that is required to get the same speed again is to set the air-valves at the previous pressure.

Working the apparatus.—The working of the apparatus is simple. The

small test-tubes (2·0 cm. × 0·5 cm.) are filled with the fluid to be centrifuged, and small rubber caps placed over the lower ends and the tubes then put in the recesses in the rotor. The top of the rotor is then placed in position, and the central screw inserted and tightened. The pressure in the stator is turned on to some 30–40 lb.; the loaded rotor, held lightly between the fingers and thumbs of both hands, is placed over the centre of the stator. Once in position it will commence to spin with increasing speed till it reaches the maximum for that pressure. The pressure is then adjusted to give the speed required, and this can be checked from time to time by the stroboscope. After the spinning is completed the pressure is reduced gradually to 20 lb., and then after a short interval, when the speed has fallen, the rotor is lifted off by means of the brass fork (Fig. 3) which has been pushed under the central steel pin of the rotor. The fork is held firmly in the hand and the rotor continues to spin for about 2 minutes. It is always well to see that there is a little grease on the fork to ensure smoother running. A mechanical device may be used for the purpose of lifting the rotor, but so far we have found the hand method very satisfactory. When the rotor has stopped the top is removed, and the tubes placed in a vertical position ready for samples to be taken. In the latter manipulation great care must be taken to avoid any shaking of the tubes and their contents. The general outlay of the apparatus is shown in Fig. 7, including the stator (B), pump lead and pressure gauges (A) and stroboscope (C).

Experimental Observations.

By means of this centrifuge a series of experimental observations have been carried out on the sedimentation times of different viruses and bacteria with the idea of obtaining their relative sizes and, if possible, some measurements of their actual size. The aim was to centrifuge these viruses and bacteria under similar conditions with, as far as possible, the number of revolutions per minute, the temperature, the nature of the suspending fluid, etc., kept constant. In the first series the revolutions were kept at 40,000 per minute, the temperature 16° C. and the standard fluid was saline. The densities of the different particles we assumed for the time being to be equal; later experiments have shown the correctness of this assumption. As already mentioned in this series, the time of spinning was either 30 or 60 minutes, except for bacteria, where the period of spinning had to be reduced to 5 or 10 minutes, as otherwise the number of bacteria was reduced to zero and an exact titration of the reduction of their numbers could not be obtained.

In Fig. 8 the earlier observations at 40,000 revolutions per minute are charted. The initial and final concentrations, expressed in powers of 10, are plotted against time, the initial concentration at zero time and the final concentration at the end of the time of spinning. When these points are joined up, the resultant line forms an angle with the top of the chart which represents the gradient of the reduction in concentration during the time of spinning. This is the angle of sedimentation (θ).

In Fig. 9 the sedimentation angles of a number of selected observations are grouped. Observations made at a speed of 40,000 r.p.m. are represented by continuous lines, and those made at 10,000 r.p.m. by broken lines (bacteria).

It is evident from a study of the figures that there is a distinct relationship between the size of the sedimentation angle and the size of the particle ; the greater the particle the greater the angle. In comparing the angles for the two spinning velocities it must be remembered that the centrifugal force is proportional to the square of the velocity, so that the fall in concentration at

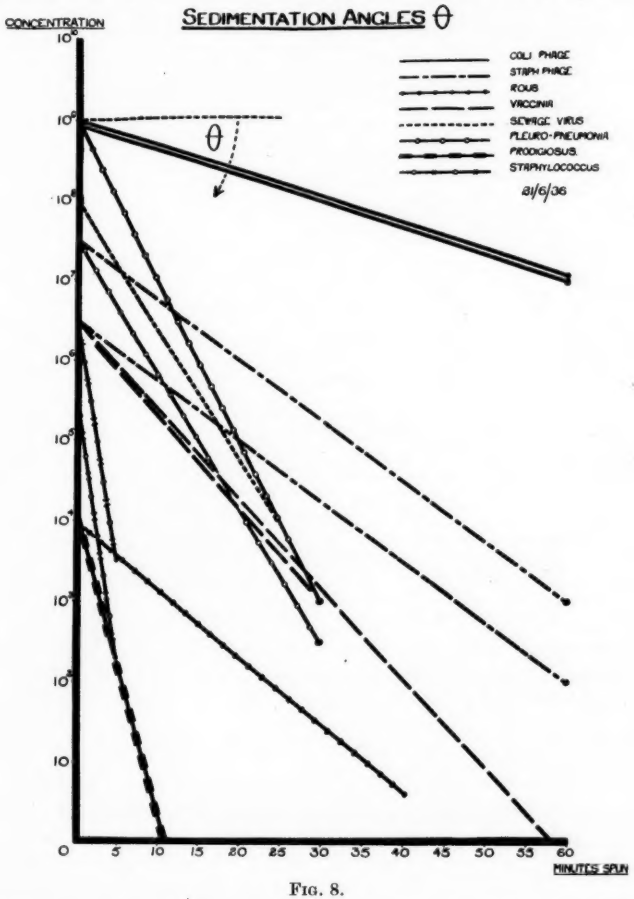


FIG. 8.

10,000 r.p.m. should be multiplied by sixteen to give the equivalent fall in concentration at 40,000 r.p.m.

Further studies of the properties of these angles show that the tangent of the sedimentation angle is a function of the velocity of the sedimentation of the particle, as it represents the ratio between the logarithmic reduction in concentration and the time of spinning. At first it seemed that a good approximation to the actual size of the particle could be obtained directly

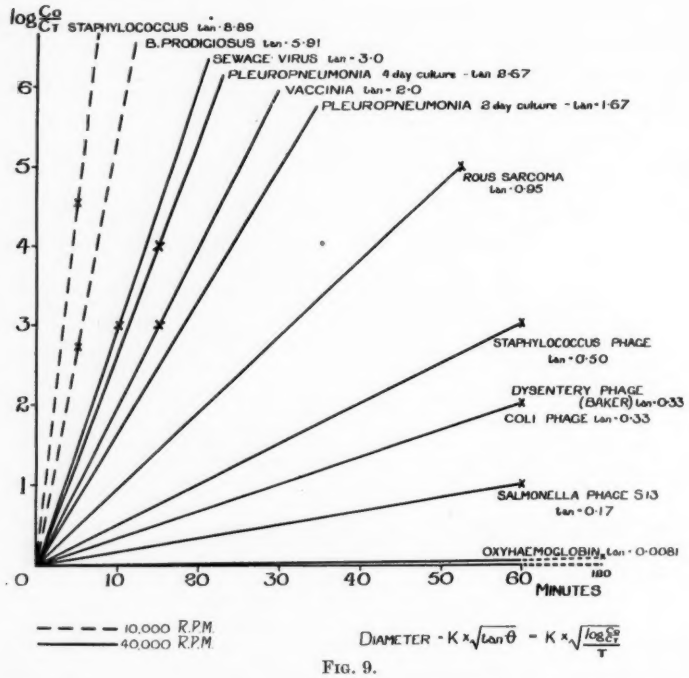


FIG. 9.

from the tangent. A further examination of the data, however (Fig. 9), showed that a still better approximation could be obtained from the square root of the tangent, and that the size of a particle could be calculated from the sedimentation angle (θ) by the following equation :

$$\text{Diameter} = K \times \sqrt{\tan \theta},$$

where K varies with the speed of the centrifuge. In order to get a value for K, a large number of staphylococci of the strain employed were measured in stained preparations by projection. The diameter was found to average 800 mμ, and we have used this value as a standard, although, by other methods of measurement, this strain has given higher averages. Thus substituting 800 mμ for the diameter and 8·89 for the tangent of the sedimentation angle θ (Fig. 9) of staphylococcus we have—

$$800 = K \times \sqrt{8{\cdot}89},$$

$$K = \frac{800}{\sqrt{8{\cdot}89}} = 268{\cdot}3 \text{ at } 10{,}000 \text{ r.p.m.},$$

$$\text{and } K = 67{\cdot}08 \text{ at } 40{,}000 \text{ r.p.m.}$$

TABLE I.

Particle.	Calculated from sedimentation angle. (mμ)	Diameter from modified Stoke's equation. (mμ)	Previous findings. (mμ)
Staphylococcus aureus	800 (direct measurement)	900	700–900 (1)
Bacillus prodigiosus	650	740	700–800 (2)
Sewage virus	116–220	135–255	125–175 (3)
Vaccinia	99–220	103–240	140–160 (4)
			170–180 (5)
Pleuro-pneumonia	97–220	103–255	125–150 (6)
Rous sarcoma	66	76	75–100 (7)
			60–70 (5)
Staphylococcus phage	47	53–55	50–75 (8)
			60–90 (9)
			60–70 (2)
B. coli phage	39	45	20–30 (8)
Shiga dysentery phage (Baker, 1929)	39	45	..
Salmonella phage S13 (anti-dysentery)	28	32	8–12 (8)
			15–17 (2)
Oxyhæmoglobin	6·1	5·6	5·6 (10)

(1) Topley and Wilson (1936); (2) Elford (1936); (3) Laidlaw and Elford (1936); (4) Levaditi, Paic and Krasnoff (1936); (5) Elford and Andrewes (1936); (6) Elford (1929); (7) Elford and Andrewes (1935); (8) Elford and Andrewes (1932); (9) Levaditi, Paic, Voet and Krasnoff (1936); (10) Svedberg (1930).

The sizes calculated by the use of this formula are given in Table I, where they are compared with those given by previous workers, and show a fairly close agreement.

As the tangent of the sedimentation angle is a multiple of $\frac{\log \frac{C_0}{C_\tau}}{T}$, where C_0 = initial concentration, C_τ = final concentration and T = time in seconds, the formula for general use would be:

$$\text{Diameter} = K \times \sqrt{\frac{\log \frac{C_0}{C_\tau}}{T}}.$$

At first sight this formula would appear to be an approximation, but more critical examination of formulæ used by others, Bechhold and Schlesinger (1931) and Elford (1936), shows that they are fundamentally the same. This constitutes the basic formula and, when necessary, suitable modifications can be made for variations in speed, viscosity, relative densities, etc.

TABLE II.—*Experimental Data.*

	Medium.	Speed (r.p.m.).	Time (minutes).	C_o.	C_τ.	Log $\frac{C_o}{C_\tau}$.	Log $\frac{C_o}{C_\tau}$. $\frac{1}{T}$
Staphylococcus aureus	Saline	40,000	5	$5{\cdot}000 \times 10^6$	$5{\cdot}0 \times 10^3$	3·000	0·6000
,,	,,	,,	5	$1{\cdot}446 \times 10^{12}$	$2{\cdot}0 \times 10^6$	5·860	1·1700
,,	,,	,,	3	$1{\cdot}650 \times 10^6$	$2{\cdot}5 \times 10^2$	3·820	1·2700
,,	,,	15,000	2	$6{\cdot}550 \times 10^5$	$7{\cdot}9 \times 10^2$	2·920	1·4600
,,	,,	10,000	5	$1{\cdot}095 \times 10^6$	$4{\cdot}0 \times 10^1$	4·440	0·8890
Bacillus prodigiosus	,,	40,000	7	$2{\cdot}000 \times 10^5$	$6{\cdot}0 \times 10^1$	3·520	0·5030
,,	,,	,,	3	$7{\cdot}850 \times 10^5$	$8{\cdot}5 \times 10^2$	2·990	0·9960
,,	,,	10,000	5	$1{\cdot}935 \times 10^6$	$2{\cdot}15 \times 10^3$	2·950	0·5910
Pleuro-pneumonia:							
48-hour culture	Serum-broth	40,000	60	$5{\cdot}000 \times 10^9$	$5{\cdot}0 \times 10^2$	7·000	0·1170
48-hour culture	,,	,,	30	$5{\cdot}000 \times 10^7$	$5{\cdot}0 \times 10^2$	5·000	0·1670
4-day culture	,,	,,	15	$5{\cdot}000 \times 10^5$	$5{\cdot}0 \times 10^1$	4·000	0·2670
48-hour culture	Broth	10,000	15	10^{10}	10^9	1·000	0·0667
Sewage virus	,,	40,000	10	10^8	10^5	3·000	0·3000
,,	,,	,,	20	10^8	10^5	3·000	0·1500
,,	,,	,,	30	10^8	10^4	4·000	0·1330
,,	,,	10,000	15	10^7	10^6	1·000	0·0667
Vaccinia	Tap-water	40,000	30	10^5	10^1	4·000	
				10^4	10^2	2·000	0·1000
				10^6	10^3	3·000	
,,	,,	,,	20	$2{\cdot}000 \times 10^6$	10^3	3·300	0·1750
,,	,,	,,	15	10^4	10^1	3·000	
				10^6	10^3	3·000	0·2000
				10^7	10^4	3·000	
,,	,,	10,000	30	10^7	10^4	3·000	
				10^4	10^2	2·000	0·0667
				10^5	10^4	1·000	
,,	,,	,,	15	10^6	10^5	1·000	
				10^6	10^5	1·000	0·6670
Staphylococcus phage	Broth	40,000	60	$10^{6{\cdot}25}$	$10^{3{\cdot}25}$	3·000	0·0500
,,	,,	,,	60	$10^{6{\cdot}75}$	10^4	2·750	0·0458
B. coli phage	,,	,,	30	$10^{7{\cdot}5}$	$10^{6{\cdot}5}$	1·000	0·0333
,,	,,	,,	60	$10^{8{\cdot}5}$	$10^{6{\cdot}5}$	2·000	0·0333
,,	,,	,,	60	$10^{7{\cdot}5}$	$10^{5{\cdot}5}$	2·000	0·0333
Shiga dysentery phage (Baker)	,,	,,	60	$10^{5{\cdot}5}$	$10^{3{\cdot}5}$	2·000	0·0333
Salmonella phage S13 (anti-dysentery, Elford)	,,	,,	60	10^4	10^3	1·000	0·0167
,,	,,	,,	60	$6{\cdot}4 \times 10^3$	$6{\cdot}0 \times 10^2$	1·028	0·0171

				Titre of filtrate.	Activity of supernatant fluid.		
Sarcoma virus (Rous)	Sand and paper-pulp filtrate	40,000	10	10^5	+		
			15		+		
			30		+	5	$5/\frac{45+60}{2}$
			45		+		
			60		−		0·0952

				$C_o : C_\tau$		
Oxyhæmoglobin	1% in saline	40,000	120	5 : 4	0·0969	0·0008075

Bechhold and Schlesinger (1932) and Elford (1936) have used modifications of Stoke's formula, from which dimensions of spherical particles can be calculated from the velocity of their sedimentation under the force of gravity:

$$v = \frac{2}{9} \frac{r^2(\sigma - \sigma^1)\, g}{\eta}.$$

where v = velocity,
r = radius of particle,
σ = density of particle,
σ^1 = density of medium,
η = viscosity of medium,
g = gravitational constant.

All in C.G.S. units.

$$\therefore \quad r = \sqrt{\frac{9}{2} \frac{\eta v}{(\sigma - \sigma^1) g}}.$$

The force applied to the particle by centrifugalization replacing g is $R\omega^2$, where ω = angular velocity $= \frac{2\pi N}{60}$.

where N = revolutions per minute,
and R = distance from the axis of rotation,

which, in the air-driven centrifuge, is 1·3 cm., being the sum of the distance from the meniscus to the centre of the rotor (1·0 cm.), and half the length of the column of supernatant fluid withdrawn for titration (0·3 cm.). (See Fig. 10.)

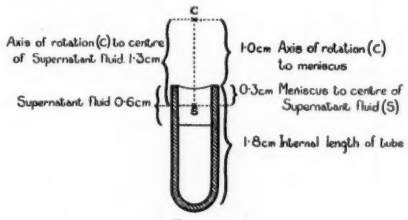

C
Axis of rotation (C) to centre of Supernatant fluid 1·3cm
Supernatant fluid 0·6cm
S
1·0cm Axis of rotation (C) to meniscus
0·3cm Meniscus to centre of Supernatant fluid (S)
1·8cm Internal length of tube

FIG. 10.

Oxyhæmoglobin was spun down into a thin layer of absorbent cotton-wool at the bottom of the tube, so that, in the calculation of the diameter of oxyhæmoglobin, $R = 1{\cdot}9$ cm., *i. e.* the sum of the distance of the meniscus from the centre of rotation and half the internal length of the tube.

Our observations show that the diameter of the particle varies directly with the square root of the tangent of the sedimentation angle, and that $\frac{\log \frac{C_0}{C_T}}{T}$ can be substituted for v (velocity).

Therefore Stoke's formula may be modified thus:

$$r = \sqrt{\frac{9\eta \log \frac{C_0}{C_T}}{2(\sigma - \sigma^1) RT\omega^2}}.$$ *

* To apply the above formula the viscosities of the various media were determined as follows:

Distilled water	= 0·01000.
Normal saline	= 0·01088.
Broth	= 0·01157.
Broth + 10% serum . . .	= 0·01204.
Rous sand and paper-pulp filtrate	= 0·01145.
Vaccinia extracts in tap-water .	= 0·01007.

The densities of the media varied from 1·0 to 1·006. The densities of staphylococcus and staphyloccocus phage were both found to be approximately 1·25 by spinning them in media of various densities and 0·25 has been used throughout for $(\sigma-\sigma^1)$.

The diameters obtained by the use of this equation are given in Table I.

It can also be appreciated from our data that our formula holds for wide values of $\log \frac{C_0}{C_\tau}$ (0·097 to 4·44), which need not be restricted within the narrow limits demanded by the authors quoted.

Estimation of Density.

The principle adopted in estimating density was to spin bacteria and viruses in fluids of different specific gravity. Some difficulty was found in obtaining a solution of sufficiently high specific gravity which had little or no injurious

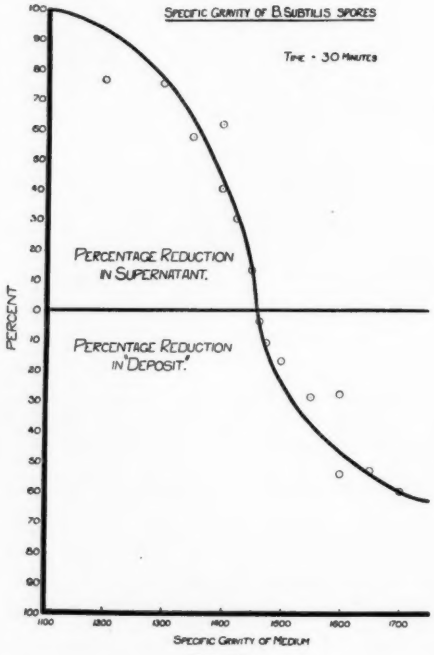


FIG. 11.

effect; we found that the fluid with least lethal action was a glucose solution, but it has the great disadvantage of a high viscosity, which makes thorough mixing of the particles difficult. For bacteria and spores we chiefly used solutions of potassium iodide. Although the method is simple in theory, in practice it was found to be difficult to get consistent readings when the fluids

were of a specific gravity approaching that of the density of the particle under observation. We found it better to use smaller tubes (diam. 0·3 cm.), and simply cut the tube in half at the end of the spinning and estimate the contents in the upper and lower halves. The percentage changes in the upper half (supernatant) and in the lower half (deposit) of the test-tube are plotted out and the specific gravity of the particle can be read off where the curve joining the percentage variations crosses the zero line (Fig. 11). When, however, the specific gravity is roughly known, only a few observations are required to determine the exact figure. By this means we have measured the specific gravity of bacteria, spores and phages. Spores were found to have the high figure of 1·46 (*B. subtilis*). Phages and *Staphylococcus aureus* gave a figure of 1·25. Our figure for staphylococcus is slightly higher than that given by other observers, but, as we have indicated elsewhere, there is room for much work yet to be done in the determination of these specific gravities.

Discussion.

It would appear that a centrifuge devised on the principle of Henriot and Huguenard provides a simple and accurate instrument for the determination of the size of viruses. This centrifuge is an instrument of precision in that it is capable of giving a constant and known centrifugal pull, which is so important in these determinations. Probably of equal importance is the fact that it runs without vibration and at a constant temperature. Other factors, such as variations in the specific gravity or viscosity of the suspending fluid or the densities, can be allowed for. No real adjustment, however, can be made for the inaccuracy associated with biological assays, and this is common to any biological test and, of course, applies equally to measurements by filtration. Any inaccuracy which may occur in the measurements must be largely due to this.

In our observations we have assumed that the viruses are spheres or spheroid in shape, and the results go to confirm this view. The ease with which it has been possible to find a formula which covers a wide range of values indicates that the conditions of sedimentation are ideal.

In certain of the observations, and particularly in the case of vaccinia, pleuro-pneumonia and sewage virus, it has been noted that the value of $\frac{\log \frac{C_0}{C_T}}{T}$ diminishes with increased time of spinning. We interpret this as an expression of variations in the size of the particles, as the larger bodies come down more rapidly. This is well shown in our data, where we have spun vaccinia at 10,000 and at 40,000 revolutions per minute and old and young cultures of pleuro-pneumonia. But in any case the titration of the supernatant fluid after a prolonged spinning is bound to be less accurate owing to the small number of particles remaining, and for that reason we have avoided prolonged spinnings. It would appear from our results that the diameters of the smaller particles (phages, etc.) computed by centrifugalization are larger than that which has been previously estimated by filtration methods.

In determining specific gravities it is to be noted that the observation must be made as expeditiously as possible, so as to limit to the smallest amount any possible interchange between the protoplasm of the virus and the suspending fluid, which is frequently far from being isotonic. Another difficulty arises from the fact that sugar solutions of high densities have a greatly increased viscosity. Salts of heavy metals, although of much less viscosity, cannot be used for viruses owing to their lethal effects. An interesting point came out in the spinning of spores in high concentrations of potassium iodide, namely, that the lethal effect of the potassium iodide was greatly enhanced by the actual spinning; spores showed normally a high resistance to the destructive effects of potassium iodide.

Summary.

Details are given for the construction of an air-driven centrifuge devised for the sedimentation of viruses and bacteria; this machine is capable of speeds of over 60,000 revolutions per minute and runs smoothly at even temperature.

The sedimentation rates of various bodies and viruses can be expressed graphically and show a "sedimentation angle", and the size of the particles has been shown to be proportional to the square root of the tangent of that angle.

$$(D = K \times \sqrt{\tan \theta}.)$$

From the data obtained a simplified formula has been constructed for determining the actual size of the particles applicable to the air-driven centrifuge. The essential similarity of the formula to that of Stokes is demonstrated.

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ERRATA.

Vol. XVIII.

Page 2, line 31.—"with exanthem" *should read* "without exanthem."

Page 23, line 4.—"Hurst, 1936*c*" *should read* "Hurst, 1937*b*".

Page 23, line 20.—"Hurst, 1936*b*" *should read* "Hurst, 1937*a*".

Page 24, line 6.—"Hurst, 1936*c*" *should read* "Hurst, 1937*b*".

Page 25.—The footnote to the asterisk on line 1 is found at the bottom of Table IV and should be at the bottom of the page. (The footnote beneath Table III refers to both Tables III and IV.